# The Effect of Dynamic Shear Force on Chondrocyte Biosynthesis in Agarose Gels

by

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B.S. Electrical Engineering
Georgia Institute of Technology, 2003

SUBMITTED TO THE DEPARTMENT OF
ELECTRICAL ENGINEERING AND COMPUTER SCIENCE
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE IN
ELECTRICAL ENGINEERING AND COMPUTER ENGINEERING

AT THE
MASSACHUSETTSETTS INSTITUTE OF TECHNOLOGY

JUNE 2005





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Submitted to the Department of Electrical Engineering and Computer Science
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ABSTRACT

The dense extracellular matrix (ECM) within the articular cartilage derives its compressive modulus from the electrostatic and osmotic interactions between highly charged glycosaminoglycan (GAG) chains. Furthermore, the structural integrity of the ECM comes from the tightly interwoven collagen network, which accounts for the tensile and shear stiffness of the cartilage. Due to the avascular, aneural, and alymphatic nature of cartilage, its native cells (chondrocytes) have limited regeneration capabilities after injury. Previous investigators discovered that dynamic compressive loading can increase cell biosynthesis in cartilage explants as well as in hydrogels such as agarose and self-assembling peptides. Also, recent research has revealed that biosynthesis in cartilage explants can be increased by dynamic tissue shear loading as well. Thus, the purpose of this study was to investigate whether dynamic tissue shear loading could affect chondrocyte biosynthesis in agarose gels.

Chondrocytes from 1-2 week old bovine knee cartilage were seeded into 3% agarose gel slabs, and 2-mm thick by 4-mm diameter cylindrical disks were cored from these gels. Groups of disks were maintained in free swelling control culture, or subjected to 5% static compression control, or 3% sinusoidal shear strain (0.1Hz) superimposed on a 5% static compressive offset. After loading, specimens were examined to measure total GAG concentration and DNA content (cell number) over the days of culture + 24 hours of loading. However, the rate of GAG synthesis using $^{35}$S-sulfate and the rate of protein synthesis using $^{3}$H-proline were only observed for the 24 hours of loading.

We found that on any given day during a time course study, dynamic shear loading caused a statistically significant increase in chondrocyte biosynthesis when compared to the controls. The data suggests that the effect of dynamic shear loading on biosynthesis can be optimized by considering factors such as the state of the cell-gel construct, the supplements added to the culture medium, and the shear loading protocol. In conclusion, the experiments, presented in this study, show that dynamic tissue shear loading, which isolates mechanical deformation from fluid flow, also has the potential to stimulate chondrocyte biosynthesis of GAG and protein in tissue engineered hydrogel scaffolds.

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# Acknowledgements

First and foremost, I would like to express my deepest gratitude towards my advisor, Dr. Alan Grodzinsky, for his encouragement and guidance. Using a unique and modern style, he encourages by reassuring students his confidence in their research prospects, and uplifting their spirits with his enthusiasm. Each day, you will always be greeted with the sound of laughter, the smell of coffee, and the sight of happy faces. Furthermore, Dr. Grodzinsky is a professor who guides through his compassion for students. For instance, his lectures are well refined each year in order to improve the learning experience. Also, even if he has a million tasks on the agenda, he always grants full attention to students regardless of the time. True, one can find countless professors who have successful careers, but it is a rare occasion to find one who also exhibits humbleness and selflessness with an air of sophistication. Therefore, it is truly an honor to be part of this great research group!

In addition, I would like to thank Dr. Delphine Dean for her words of wisdom regarding research and academics. Next, I want to thank Laurel Ng, Diana Chai, and Jonathon Fitzgerald for being so thorough in answering all my questions. Moreover, I want to thank Dr. Michael DiMicco, Dr. Bernd Rolauffs, and Dr. John Kisiday for sharing with me insightful knowledge on the latest research. Bravo to Dr. Elliot Frank and Ms. Han-Hwa Hung for providing laboratory training and playing important roles in our education. And, much thanks to Ms. Linda Bragman for her delightful humor and helpful solutions to administrative questions. Above all, cheers to everyone in the Continuum Electromechanics Group for their friendship and creating such a fun working atmosphere!!!

The origin my education and development lies in my family. So, I want to take a moment to thank my paternal grandparents for taking care of me since day 1 and instilling in me the confidence to strive towards my goals. Simultaneously, I want to thank my parents for their years of hard work and sacrifice so that my sister and I can have a healthy and fulfilling life to build our dreams upon. In addition, I want to show appreciation towards my youngest aunt in Shanghai for raising me like her own when I was in China. Of course, I must mention my little sister, whom I absolutely adore because she is the reason for my neverending childhood.

The MTL open house opened my eyes to the wonders of MIT when I first visited, so I would like to thank Ms. Debb Hodges-Pabon for hosting the unforgettable event. Also, I want to thank Ms. Carolyn Collins for her friendship and support over the past two years. Last, but not least, I want to sincerely thank Ms. Xiaoman Duan for showing me the key to happiness is to make other people happy.

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# List of Abbreviations

| | |
|---|---|
| * | Statistically significant ($p<0.05$) |
| **ACL** | Anterior Cruciate Ligament |
| **AFM** | Atomic Force Microscope |
| **DMEM** | Dulbecco/Vogt modified Eagle's Minimal Essential Medium |
| **DMMB** | dimethylmethylene blue |
| **ECM** | Extracellular Matrix |
| **FBS** | Fetal Bovine Serum |
| **GAG** | Glycosaminoglycan |
| **HEPES** | N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| **IGF-I** | Insulin-like Growth Factor I |
| **ITS** | Insulin, Transferrin, and Selenium |
| **NEAA** | Nonessential Amino Acid |
| **PBS** | Dulbecco's Phosphate-Buffered Saline |
| **PCM** | Pericellular Matrix |
| **PG** | Proteoglycan |
| **PSA** | Penicillin and Streptomycin |

# Chapter 1

# Introduction

## 1.1 Articular Cartilage

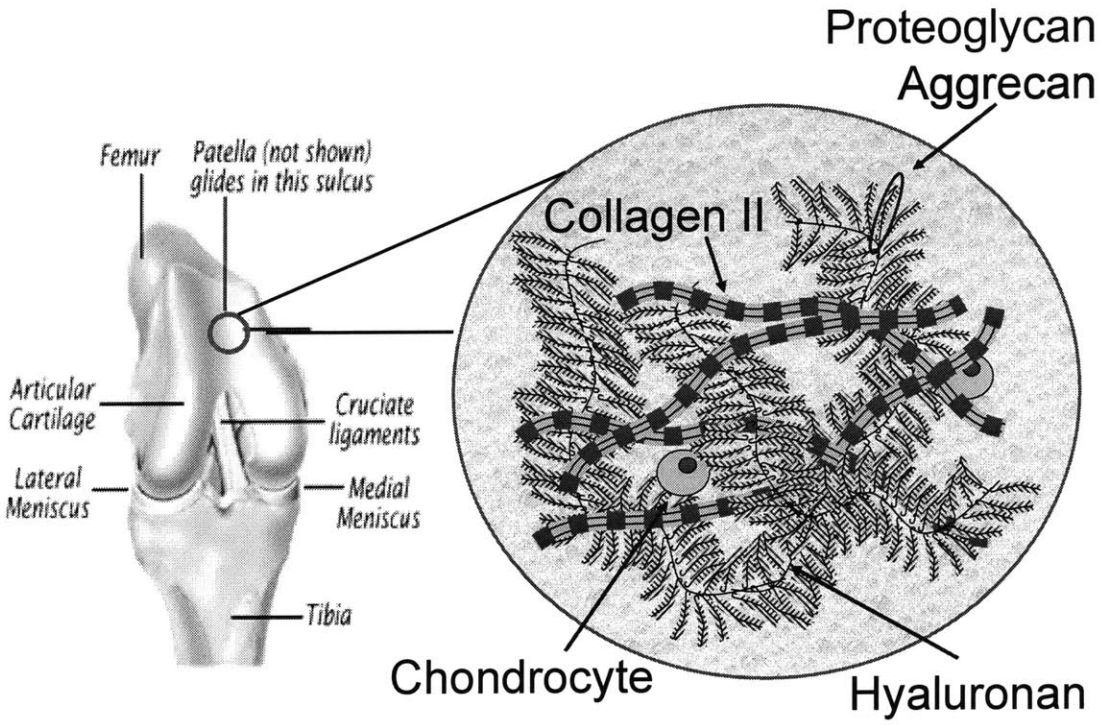



**Figure 1.1** Diagram of knee joint (left) and cartoon of detail components inside the articular cartilage (right). [The respective images are from www.wnycrc.buffalo.edu/learnknee.html, and *Dr. Delphine Dean.*]

Articular knee cartilage, shown in Figure 1.1, is the essential load bearing connective tissue that shields the surfaces of the tibia (shin bone) and femur (thigh bone) from impact and wear during daily activities. Inside, cartilage cells (chondrocytes) are encased within an extracellular matrix (ECM) composed of collagen-II protein fibrils, and aggregating

proteoglycans (aggrecan). These aggrecan molecules bind non-covalently to hyaluronan chains, stabilized by link protein [1]. Together, the dense ECM forms a stiff sponge with a water content of 75~80% [2]. The rigidity of the cartilaginous tissue results in part from electrostatic repulsive forces between highly charged proteoglycan macromolecules tightly interwoven into the collagen network. These elements, synthesized by chondrocytes, play principal roles in establishing the mechanisms which influence the macroscopic properties of cartilage [2].

### 1.1.1 Chondrocytes

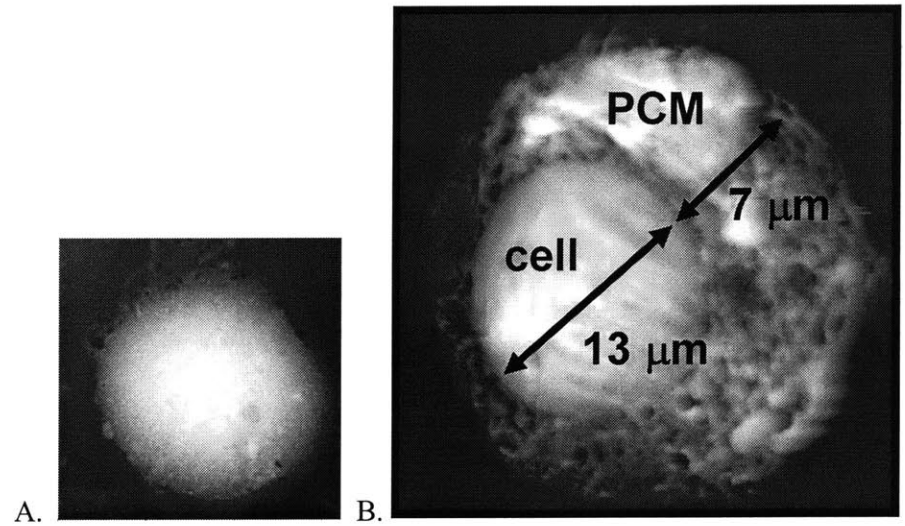



**Figure 1.2** AFM images showing (A) a single chondrocyte on day 0 and (B) a single chondrocyte surrounded by a layer of pericellular matrix on day 11. [*Curtesy of Laurel Ng*]

Chondrocytes, shown in Figure 1.2, are the focus of this study because they are responsible for continuously synthesizing, assembling, and turning over matrix proteins inside the cartilaginous tissue. During development, chondrocytes first assemble a pericellular matrix characterized by the presence of collagen-VI. In mature cartilage, this cell associated matrix forms a chondron, which is surrounded by a dense ECM, and which defines the cell's mechanical environment on a microscale [3]. Chondrocyte biosynthetic activities can be

regulated by their surrounding mechanical environment due to external loading conditions. The specific signaling pathways that lead chondrocyte to sense and respond to mechanical loading are not fully understood.

### 1.1.2 Aggrecan

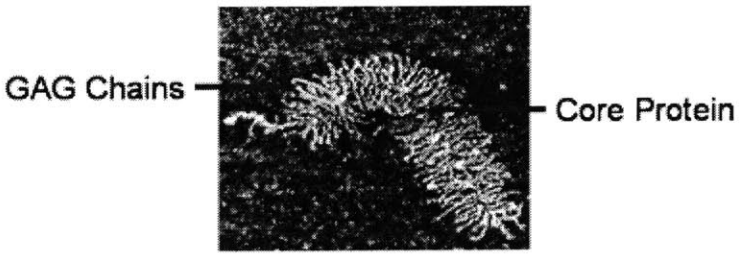



**Figure 1.3** AFM image of a single aggrecan [4].

Aggrecan is the predominant member of proteoglycan family that exists in cartilage. Zooming in on a nano-scale, the AFM image of aggrecan reveals that there are approximately 100 highly charged GAG chains that aggregate along the core protein backbone. The negative sulfate and carboxyl side groups on each of the ~50 repeating GAG disaccharide units, shown in Figure 1.4, contribute to the electric repulsion force that allows the extended chondroitin sulfate GAG chains to be modeled like rods [5].

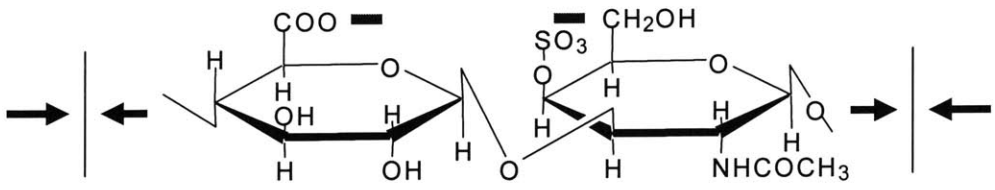



**Figure 1.4** A negatively charged repeating disaccharide unit on the GAG chain.

From Figure 1.1, we also know that these aggrecans form super-aggregate assemblies by further bonding noncovalently to hyaluronan molecules, stabilized by link proteins. Moreover, by attracting counter-ions to the fixed charges, PG macromolecules create a local osmotic imbalance. Together, these osmotic and the electrostatic repulsion interaction from the hierarchical arrangement of polyelectrolyte brushes help to counteract against external compressive loading [6].

### 1.1.3 Collagen

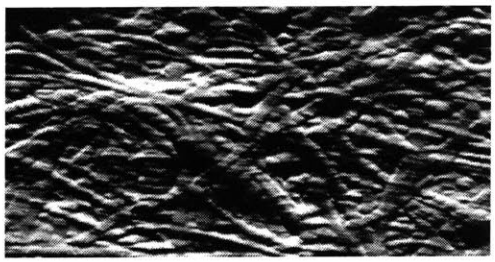


**Figure 1.5** AFM image of the collagen network. [Curtesy of Laurel Ng]

The collagen network, responsible for over two thirds of the dry weight in ECM, is a cross-linkage of collagen-II, IX, and XI. Although there are many types of collagen proteins, the triple helical collagen-II fibrils remain the most dominant component. The primary functions of the rigid framework are to maintain structural integrity and hold the cartilage in tact. Furthermore, the alignment of the fibrils when pulled allows the cartilage to withstand tensile and mechanical shear force [7].

## 1.2 Biomechanical Regulation of Matrix Biosynthesis in Explants

There is a mutual dependency between the biomechanics and the biosynthesis of the matrix elements within the cartilage. The intricate interactions that take place on a molecular level amongst aggrecan, collagen, water, and other elements govern the mechanical properties of the cartilage. At the same time, cellular response to tissue-level external loading helps to regulate the cellular activities that maintain cartilage homeostasis. Such external mechanical forces include dynamic compressive and shear loading have been shown to stimulate chondrocyte biosynthesis in the matrix. However, there is yet no complete characterization of the signaling pathway by which mechanotransduction (the translation of mechanical signals to biochemical signals) regulates cellular processes *in vivo*.

### 1.2.1 Static and Dynamic Compressive Loading

The equilibrium compressive modulus of cartilage tissue is approximately 500~800 KPa [8], while the dynamic compressive modulus has been shown to be around 13~37 MPa when 0.5~2.5% strain amplitude is applied at 1 Hz [9-11]. Furthermore, previous research has indicated that applying dynamic compressive loading to cartilage explants enables a coupling between dynamic fluid flow and slight matrix deformation within the tissue [10]. In turn, that can enhance the exchange of soluble factors. The combination of mechanical signals and enhanced transport can increase the biosynthetic activity of cells localized near the peripheral region of cylindrical bovine cartilage explants [11, 12]. In fact, oscillatory compressive strain between 1~5% at 0.01~1 Hz can increase the sulfate and proline incorporation, indicative of GAG and protein synthesis, respectively, by 20~40% after just 24 hours of loading [9].

### 1.2.2 Dynamic Tissue Shear Loading

Dynamic tissue shear loading decouples mechanical deformation from fluid flow because shear force produces little amount of volumetric deformation. Previous findings show that application of dynamic tissue shear loading can increase protein and proteoglycan synthesis by ~50% and ~25%, respectively, in cartilage explants at frequencies between 0.01 and 1.0 Hz and shear strain amplitude of 3%. Thus, one of the speculations from that study is dynamic shear loading favors protein synthesis over proteoglycan synthesis [13].

## 1.3 Tissue Engineering of Articular Cartilage

### 1.3.1 Physiological Limitations of Cartilage

Due to the avascular, aneural, and alymphatic nature of cartilage, it has limited self-healing capabilities once injured, degraded, or subjected to disease. Additionally, the decrease in chondrocyte proliferation and biosynthesis as the tissue matures also contributes to the inability of cartilage to mend any defects. Thus, ~80% of ACL (knee) injuries lead to osteoarthritis within 13~14 years [14]. Therefore, this problem motivates intense tissue engineering research in seeking techniques for cartilage repair or replacement.

### 1.3.2 Tissue Engineering Materials

Although a direct approach to this problem would be to inject fresh chondrocytes into any voids to allow it to self repair, there are many clinical challenges [15]. A better solution would be to encapsulate chondrocytes within a 3-D biodegradable scaffold that is also compatible with its surrounding environment. That way, the newly synthesized ECM can replace the scaffold

when the neocartilage has fully developed. Along with that, cell proliferation and pheonotypic expressions are also being carefully considered when selecting a scaffold. Throughout the tissue engineering field, a series of polymeric and hydrogel materials are being explored as potential scaffolds to cartilage repair [16]. Among these are two materials used in this lab: alginate and self-assembling peptide gel [17]. In addition, chondrocyte-seeded agarose gels have been used by cartilage biologists and tissue engineers as a critically important model system for tissue engineering.

### 1.3.3 Effects of Mechanical Loading on Agarose-Chondrocyte Construct

Because the scaffold construct chosen for this study is agarose gel, previous work detailing the respective effects of dynamic compressive loading and tissue shear loading on agarose-chondrocyte construct will be discussed. In a recent study by Mauck et al., the equilibrium modulus for the cell-gel culture significantly increased after undergoing dynamic compressive loading after loading intermittently at 1 Hz with 10% strain amplitude [18]. Also, Buschmann et al. discovered that the effectiveness of dynamic compressive loading on chondrocyte biosynthesis depended upon the amount of matrix already deposited around the cells. By the way, the loading protocol applied on the samples was 10 hr of continuous dynamic compression at 1 Hz with 3% strain amplitude [19]. Furthermore, Kisiday et al. conducted a series of long term experiments on chondrocyte-agarose and -peptide scaffolds in order to find the optimal duty cycle for matrix biosynthesis. As it turns out, applying intermittent loading on alternate days for long-term cultures significantly increase the sulfate incorporation on a given day [20].

Similarly, studies have also been done regarding the effect of tissue shear loading on mechanical properties of chondrocyte-agarose construct. For instance, Knight et al. developed a system for applying static tissue shear force on the construct while it is held between two porous glasses. The goal was to image the mechanical strain fields when the construct has been subjected to a ramped shear deformation [21]. Meanwhile, Raimondi et al. observed the equilibrium shear modulus in the construct when dynamic shear loading has been applied [22]. Finally, Waldman et al. performed a study showing that long term shear loading during culturing of chondrocyte-gel construct increased the proteoglycan content by 35% and collagen content by 40% compared to controls over the course of four weeks [23].

## 1.4 Objective

The purpose of this study is to quantify the effect of applied dynamic shear loading on chondrocyte biosynthesis in 3-D agarose gel scaffolds. More specifically, a series of experiments has been conducted to examine how 24 hours of continuous dynamic shear loading will affect the matrix biosynthesis compared with free swell control and 5% static compression control. Drawing from previous findings regarding the effect of dynamic compression on cell-gel constructs and dynamic shear force on cartilage explants, the hypothesis is that dynamic shear can also increase matrix biosynthesis by chondrocytes in agarose gel culture.

# Chapter 2

# Methods

## 2.1 Chondrocyte Isolation and Encapsulation in Agarose Gel

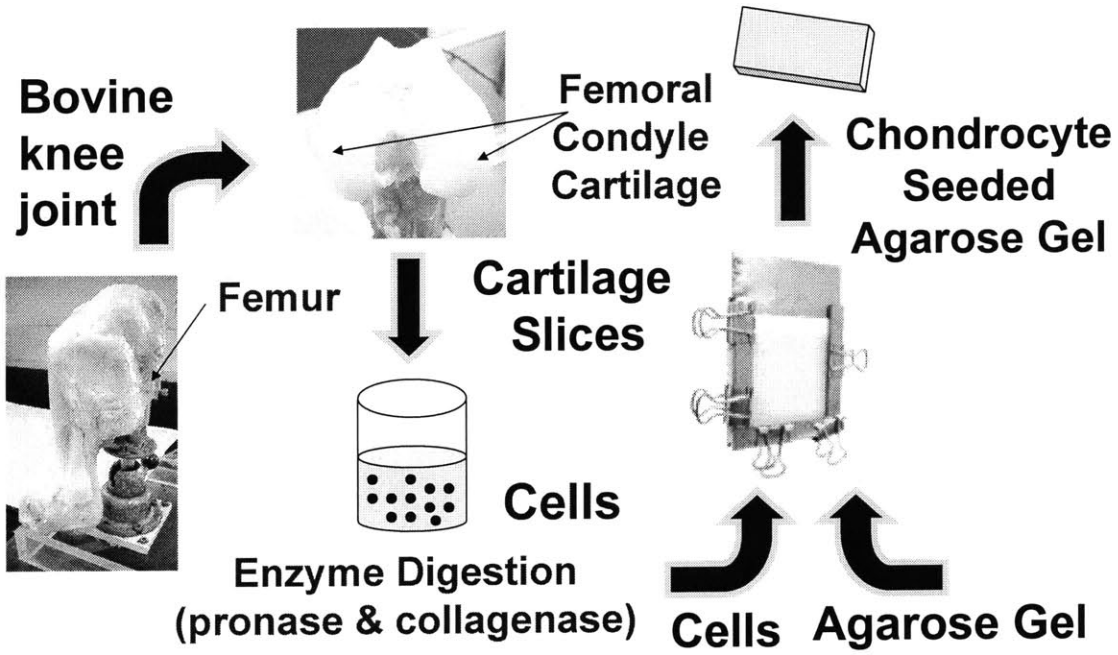



**Figure 2.1** General diagram of chondrocyte isolation and encapsulation in gel.

### 2.1.1 Cartilage Extraction

The five experiments conducted for this study began with harvesting cartilage tissues from six 1-2 week old bovine knee joint obtained from Research 87 (Boylston, MA). Under sterile conditions, the tibia (shin) was fully detached from the joint by severing the connecting ligaments and meniscus to reveal the femur condyles. Then, thin slices of cartilage tissues were shaved off the condyles surface layer and cleansed twice using 1% PSA supplemented PBS

before being transferred into a spinning flask for enzyme digestion. A pictorial description of this process is shown above in Figure 2.1.

### 2.1.2 Enzyme Digestion

First, cartilage slices were digested in 0.2% pronase solution (10ml/gram of tissue) for 2 hours in a 37° C incubator with 5% $CO_2$. The pronase was used to remove matrix components such as aggrecan to allow the collagenase-P to penetrate through to breakdown the collagen fibrils. Next, approximately the same amount of 0.025% collagenase-P solution was added to the cartilage slices for overnight digestion in the incubator. Note that both the pronase and the collagenase solution include the stock culture media (High glucose DMEM, 0.1mM MEM Non-Essential Amino Acid (NEAA), 1mM sodium pyruvate, 10mM HEPES buffered solution, 100U/ml penicillin and 100 μg/ml streptomycin, and 0.4mM proline) supplemented with 5% FBS.

### 2.1.3 Cell Isolation

The next day, the cell solution was filtered through a 70 um cell strainer prior to being spun down in 20°C centrifuge for 8 minutes at 2000 rpm. Next, the cell pellet was washed twice in PBS before suspending in fresh culture medium with 1% ITS (10 μg/ml insulin, 5.5 mg/ml transferring, and 5 ng/ml sodium selenite). The resulting cell solution was stored in 4 °C refrigerator.

### 2.1.4 Cell Counting

In order to obtain a concentration suitable for counting under the microscope, the cell solution was diluted with PBS in a 1:4 ratio and with Trypan Blue in a 1:1 ratio. Afterwards, 10

ul of the final diluted cell solution was inserted between a cover slip and the hemocytometer for counting. The final estimated cell count was derived from averaging the cell number in the four quadrants.

$$\text{Total } \frac{\text{cells}}{\text{ml}} = \text{number of } \frac{\text{cells}}{\text{mm}^3} \times 1000 \frac{\text{mm}^3}{\text{cm}^3} \times 5 \times 2 \text{ (dilution factor)} \times (0.1 \text{ ml})^{-1}$$

### 2.1.5 Casting Process

The casting process was conducted using a 2-mm thick casting frame to obtain a 3-ml slab of agarose gel with a cell seeding density of ~15 million/ml. First, the materials displayed in Figure 2.2A were assembled in the following manner: mesh and the filter paper were carefully placed on either sides between the U-clamps and the casting frame with the mesh facing the outside. Then, five stainless steel paper clips were strategically fastened around the setup to secure the apparatus, as shown in Figure 2.2B.

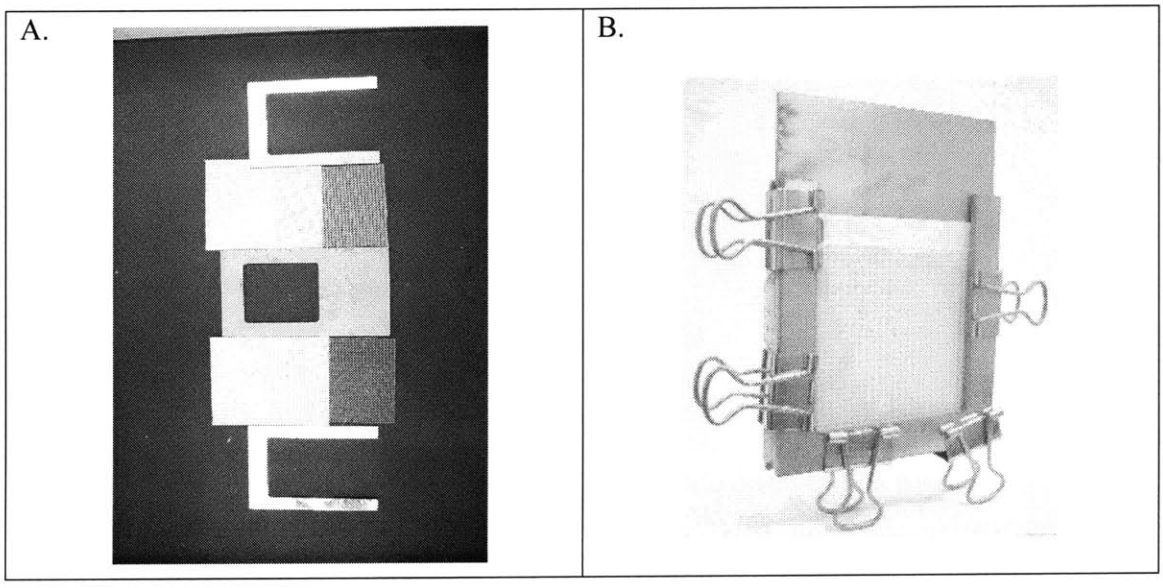



**Figure 2.2** A) Layout of all the parts in the casting apparatus including the casting frame in the middle surrounded by filter papers, meshes, and the two U-clamps. B) Assembled casting apparatus.

Since the target seeding density was 15 million/ml, the total number of cells used per slab was 45 million for a 3-ml gel. Additionally, the proportion between 3% agarose gel and cell solution was 2:1 in order to attain a 2% gel with 15 million/ml cell density. In practice, 2 ml of 3% agarose was added to 1 ml of high glucose DMEM containing the 45-million cell pellet. Immediately after, they were quickly vortexed before solidifying in the casting apparatus, as shown in Figure 2.1. Finally, the newly formed chondrocyte cell-gel slab was incubated in the culture medium at 37 °C with a medium change every other day. See Table 2.1 in Section 2.3 for more details on the culture medium used for each experiment.

## 2.2 Mechanical Loading

On the day of loading, 10μCi/ml of $^{35}S\text{-}SO_4^{-2}$ and 20μCi/ml of $^{3}H$-proline were supplemented to the culture medium (see Table 2.1 in Section 2.3) subsequently for determination of newly synthesized GAG and protein biosynthesis, respectively. Meanwhile, 0.5-ml samples of $^{35}S\text{-}SO_4^{-2}$ and $^{35}S\text{-}SO_4^{-2}$ + $^{3}H$-proline were taken as standards for spillover and media radioactivity normalization.

Then, equal number of cylindrical disks measured 4-mm diameter x 2-mm thick, extracted from the slab, were placed into the following loading conditions for 24 hours of continuous loading: free swelling control, 5% static compression control, and dynamic shear strain + 5% static offset at 0.1 Hz. A diagram explaining how the loading conditions were imposed on the disks is shown in Figure 2.3. The offset static compression value was designated at 5% in order to provide traction between the agarose gel and the platen on the lid of the shear chamber without causing too much deformation. The reason for selecting 0.1 Hz was because it was within the range of frequency (0.01 Hz ~ 1 Hz) used in dynamic compression that have been proven to stimulate matrix biosynthesis.

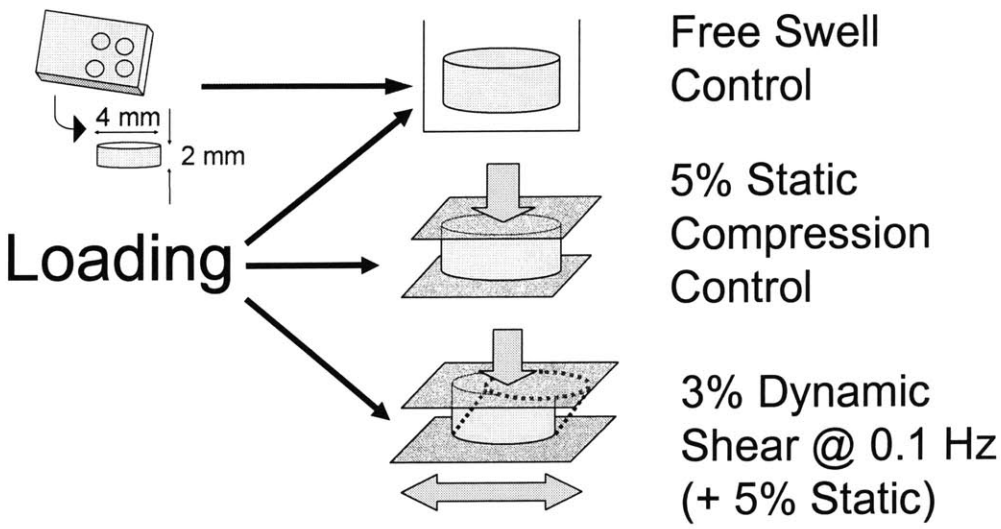



**Figure 2.3** Conceptual overview of the mechanism behind the different loading conditions.

### 2.2.1 Free Swell Control

Initially, a few wells in the 48-well plate were filled with 0.5 ml of radiolabeled media. Then, after the disks were extracted by a 4-mm punch, they were carefully transferred to those wells, as shown below in Figure 2.4.

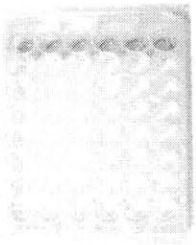


**Figure 2.4** Cylindrical disks resting in the top six wells containing radioactive medium under free swell condition.

### 2.2.2 Static Compression Control

Upon setting the disks into the individual indentations in the middle of the wells, the lid was gently closed over the chamber with separators in the middle. The total thickness of the

separators was 1.90-mm in order to compress the disks by 0.1 mm (5% of 2-mm disk). After making sure that the disks were settled properly in the middle of the indentation, the entire chamber was firmly clamped down on a stainless steel square base with some nuts and bolts, shown in Figure 2.5C. Finally, 0.5 ml of radiolabeled media was injected through the small openings into the wells.

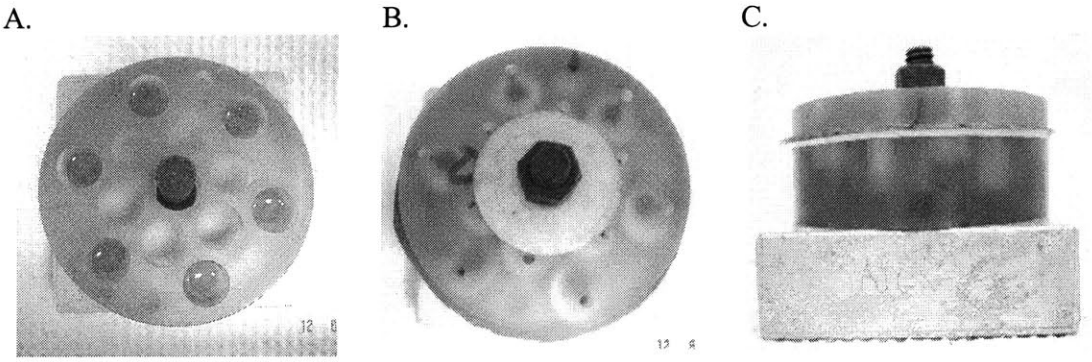



**Figure 2.5** (A) Top view of static chamber with disks in radiolabeled solution. (B) Top view of assembled static chamber with the disks in 5% compression. (C) Side view of the assembled chamber. The static chamber was made from polysulfone. At the bottom of each well, there is an indentation of 250μm deep where the sample would be placed. Furthermore, since there is a 250μm gap between the lid and the base and the platen cannot reach into the indentation, a 250μm separator lying in between the chamber or no separator would produce the same amount of static compression, which is 75%.

## 2.2.3 Dynamic Shear Loading

After settling the disks in the indentations of the shear chamber, 500 μl of radiolabeled media was carefully introduced around each disk without breaking the adhesion between the pipette tip and the fluid. This delicate process is illustrated in Figure 2.6.

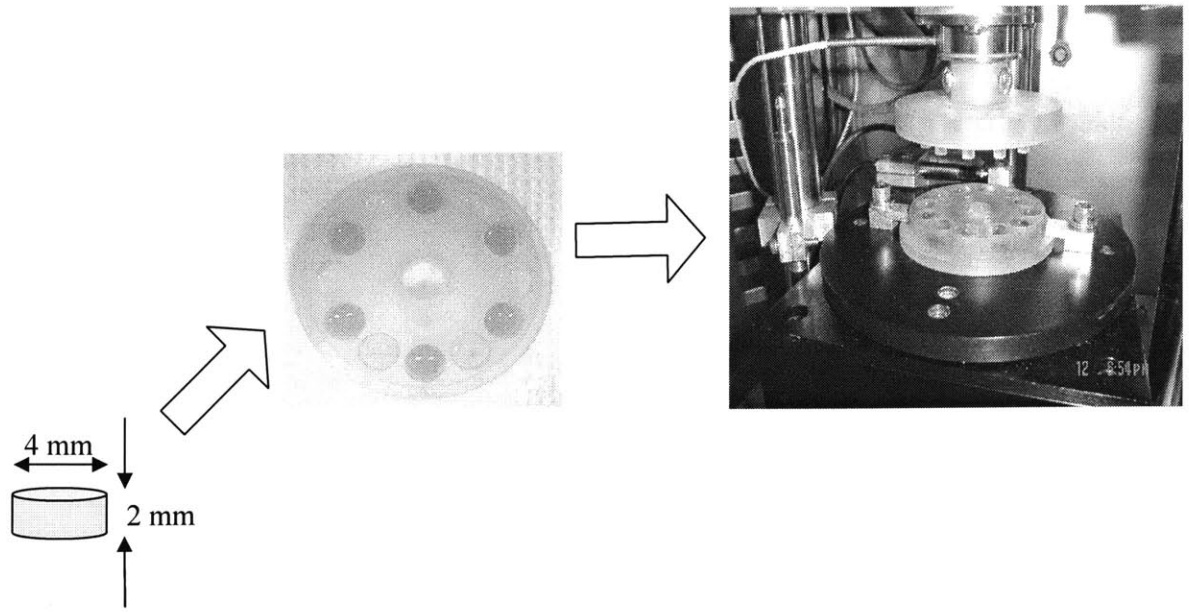



**Figure 2.6** The steps involved in transferring the disk to the shear chamber and setting it up in the incubator housed loading apparatus.

Then, as shown in Figure 2.6, the lid was fastened to the torque cell while the chamber was locked to the rotating base of the incubator housed loading apparatus. Next, the lid was lowered unto the chamber until the platen and the disks made contact. The contact point, marking the position of the 0% offset, was determined according to the graph below, Figure 2.8.

The vertical displacement on the left y-axis of the calibration curve was measured by the displacement sensor shown in Figure 2.7. Additionally, the compressive load indicating the total force felt by all the disks was measured from the load cell on the top of the machine. Once the displacement position of the full contact point was established, the lid was brought down another 100 μm from that point for the 5% offset. Then, the disks were subjected to 1 or 3% sinusoidal shear strain, measured by the angular displacement sensor, at 0.1 Hz.

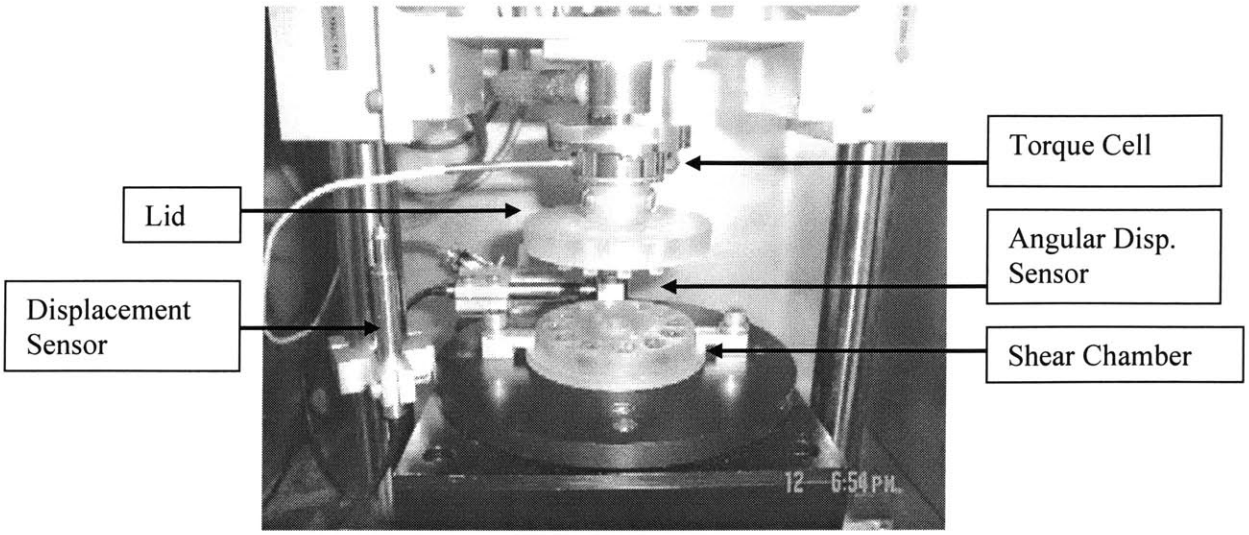



**Figure 2.7** The shear chamber in the incubated housed loading apparatus.

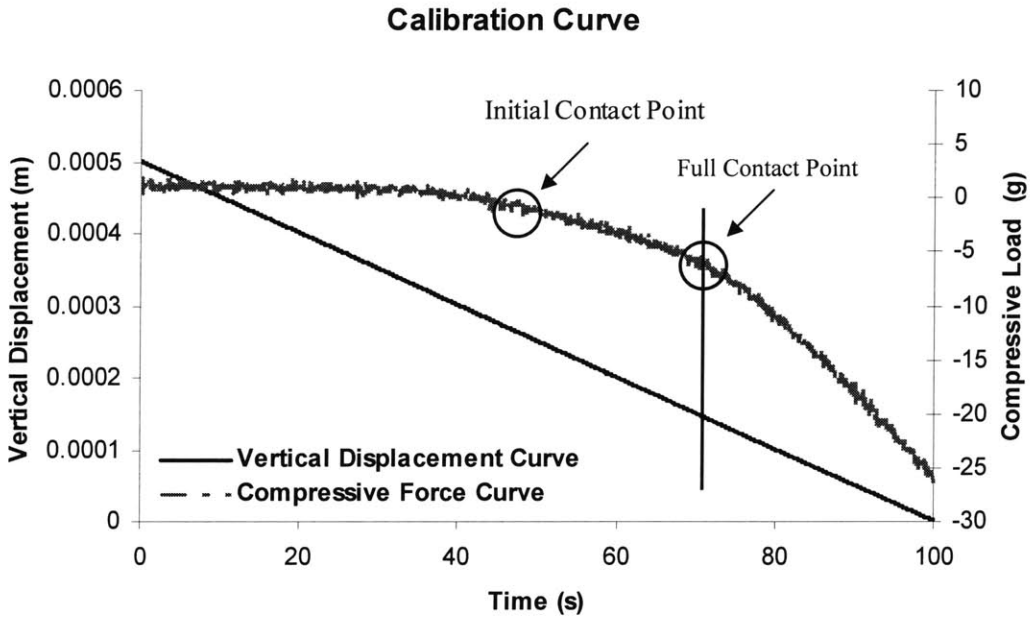



**Figure 2.8** The caliberation curve used to establish when the platen has made contact with the disks. Since the platen cannot make contact with all the disks simultaneously, the initial contact point indicates when the platens have first touched some of the disks and full contact point is when there is full contact between the disks and the platens.

## 2.3 Specific Experimental Loading and Culture Parameters

A total of five experiments using five different 1-2 week old bovine joints were conducted to analyze how 24 hours of continuous shear loading affected the chondrocyte biosynthesis after certain days of culture (loading day). Table 2.1 below provides a summary of the experimental conditions used.

**Table 2.1** Summary of the Five Experimental Conditions

| Preliminary Experiments | Loading Day | Shear Strain Amplitude | Culture Medium Supplement | # of Samples per Loading Condition |
|---|---|---|---|---|
| I | 4-5 | 1% | 10% FBS | 12 (Total n=24) |
| II | 6-7 | 1% | 1% ITS | 12 (Total n=36) |
| | 14-15 | 1% | 1% ITS | 12 (Total n=36) |
| III | 7-8 | 3% | 1% ITS | 6 (Total n=18) |
| | 12-13 | 3% | 1% ITS | 6 (Total n=18) |
| | 24-25 | 3% | 1% ITS | 6 (Total n=18) |
| **Final Experiments** | | | | |
| IV | 6-7 | 3% | 1% ITS+0.2% FBS, 1% ITS | 6 (Total n=36) |
| | 14-15 | 3% | 1% ITS+0.2% FBS, 1% ITS | 6 (Total n=36) |
| V | 0-1 | 3% | 1% ITS | 6 (Total n=18) |
| | 2-3 | 3% | 1% ITS | 6 (Total n=18) |
| | 4-5 | 3% | 1% ITS | 6 (Total n=18) |
| | 6-7 | 3% | 1% ITS | 6 (Total n=18) |
| | 14-15 | 3% | 1% ITS | 6 (Total n=18) |

*** Culture Medium = {Culture Medium Supplement, High glucose DMEM, 0.1mM MEM Non-Essential Amino Acid (NEAA), 1mM sodium pyruvate, 10mM HEPES buffered solution, 100U/ml penicillin and 100 μg/ml streptomycin, 0.4mM proline, and 20 μg/ml ascorbic acid}.**

Experiment I was a trial experiment to see if dynamic shear force can encourage chondrocyte biosynthesis in agarose gel by comparing its effect with that of static control. Since 10% FBS was commonly used when working with cartilage explants, this was convenient supplement to use simply to see if shear force can produce any effect. However, in Experiment

II, 10% FBS was substituted by 1% ITS, used as a defined medium to substitute for serum, since the components of serum may vary and serum usage is debatable for certain applications in tissue engineering. Furthermore, experiment II explored whether the effect that dynamic shear force density has on chondrocyte biosynthesis was dependent on the existing matrix density. Thus, we chose to observe two time points: day 6, when the matrix was gradually forming, and day 14, when the matrix has begun to fill within the scaffold. Also, from the 2$^{nd}$ experiment onward, a free swelling condition was also used as a second control. Later, in experiment III, a higher shear strain rate, 3%, was used instead of the previous 1% in hopes of increasing the effect that dynamic shear loading could have on chondrocyte biosynthesis.

Even though the use of serum remains questionable in tissue engineering as discussed previously, experiment IV aimed to probe whether very low concentration of it can further stimulate chondrocyte biosynthesis in shear load samples. This experiment was motivated by a study done by Dr. John Kisiday that explored the respective effects of 10% FBS, 1% ITS, and 1% ITS+0.2% FBS on chondrocyte biosynthesis in agarose scaffold under free swelling condition [24]. Although the study showed that biosynthesis under free swelling conditions experienced negligible change when 0.2% FBS was added to 1% ITS in medium, we thought that perhaps mechanical loading could exaggerate that difference. Finally, experiment V sought to find an optimal time point where 24-hr shear loading could influence biosynthesis the most effectively. In other words, perhaps there exists a particular time where the state of the matrix and cells allows biosynthetic rate to peak compared to the other days after subjecting to dynamic shear loading. Therefore, the points selected for this time course included day 0, 2, 4, 6, and 14.

## 2.4 Biochemical Assays

Once 24 hours of continuous loading was complete, the disks were washed four times at 15-minute intervals using PBS washing solution which included sodium sulfate and 0.8mM L-proline. Later, each disk was melted and digested in 1 ml of protease-K enzyme diluted 1:20 in Tris-HCl buffer solution at 60°C.

The results were obtained from the three different chemical assays conducted once loading was complete. They included assays that measure total accumulated GAG and DNA concentration over the days of culture and 24 hours of loading, along with sulfate and proline incorporation rate over only the 24 hours of loading.

### 2.4.1 Total GAG Assay

Total GAG concentration in each disk was quantified by using the DMMB assay to evaluate the amount of sulfated GAG content in the sample. More specifically, the DMMB assay measured the sulfated GAG content by the absorbance of light at 520 nm [25]. Under ambient conditions, 20 μl was drawn from the digested sample to place in a 96 well plate. To make the result more precise, a duplicate for each sample was also taken. Soon after, GAG standards were made ranging from 100μg/ml to 0μg/ml by serial diluting in Tris buffer. Simultaneously, duplicates of 20 ul medium were also placed in in the plate to measure GAG loss into the medium. Then, 200 μl of DMMB dye was added to the samples prior to sliding the plate into the spectrometer while setting the wavelength at $\lambda$=520nm. Finally, the optical density values recorded were converted to GAG concentration by comparing with a standard curve.

### 2.4.2 DNA Assay

Unlike measuring the GAG content with DMMB assay, DNA assay is a fluorometric assay where interaction between the DNA and Hoechst dye (H33258) resulted in a fluorescent activation [26]. Since DNA concentration represents cell number, the purpose of conducting this assay was to observe any trend in cell proliferation. The protocol for the DNA assay was quite similar to the one for total GAG assay. First, duplicates of 20 μl were drawn from the sample to place in an opaque 96-well plate. Then, DNA standards were made ranging from 10ug/ml to 0 by serial diluting in Tris buffer. Immediately after, 200 μl of diluted DNA Hoechst dye was added to the samples before positioning the plate into the DNA reader to evaluate DNA concentration in each sample. Lastly, the data collected was converted to DNA concentration by comparing with a standard curve.

### 2.4.3 Scintillation Counting for Radiolabel Incorporation

The scintillation count was conducted on $^{35}$S-sulfate and $^{3}$H-proline to observe the incorporation rate of these radiolabeled molecules into the plug over the course of the 24-hr loading period. The respective incorporation rate indicates how fast GAG and protein were synthesized. Because there exists a 1:1 ratio between sulfate and the chondroitin sulfate disacharride molecule, it is safe to assume that all of the sulfates incorporated were used to synthesize GAG chains. However, according to previous findings, approximately only 70~80% of the proline were used to produce collagen-II, which is the predominant type of protein in cartilage.

To begin, an initial test on the scintillation glass vials was first ran to make certain that they contained no radioactive elements, quantified by radioactive counts <12 over a 30-second period. At the same time, duplicate samples of 100 μl digest and 10 μl radioactive standards were placed into the translucent scintillation tubes. Then, 2 ml of scintillation fluid was added into the tubes. Afterwards, the samples were taken into the scintillation count to be examined for 3 minutes each.

# Chapter 3

# Results

## 3.1 Preliminary Results

### 3.1.1 Experiment I: can dynamic shear loading affect chondrocyte biosynthesis?

The aim of the initial experiment was to explore the possible effect that shear loading might have on chondrocyte biosynthesis in agarose gel culture. Thus, after applying 24 hours of continuous loading to the agarose disks from day 4 to day 5, biochemical assays were conducted to evaluate the results. The data below reveal the average DNA concentration per disk, the total GAG accumulation retained in the plug throughout the culture period + 24 hours of loading, along with sulfate and proline incorporation rates during the 24 hours of loading.

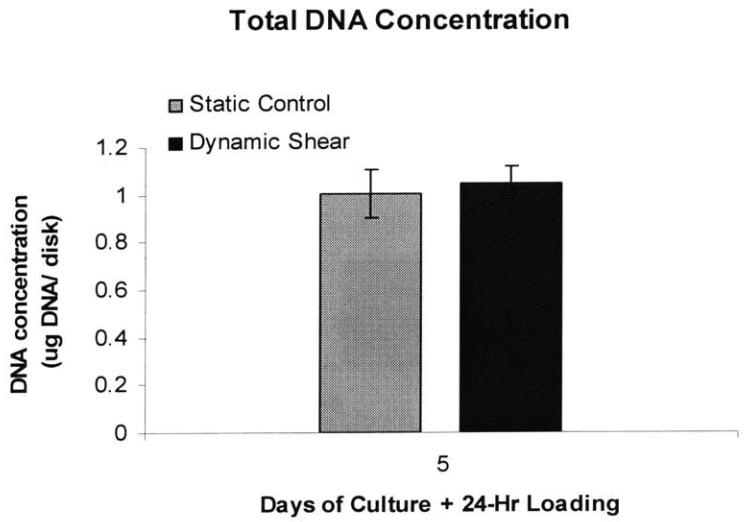



**Figure 3.1** The average total DNA concentration for one disk throughout the given days of culture + 24-hr loading. (n=12)

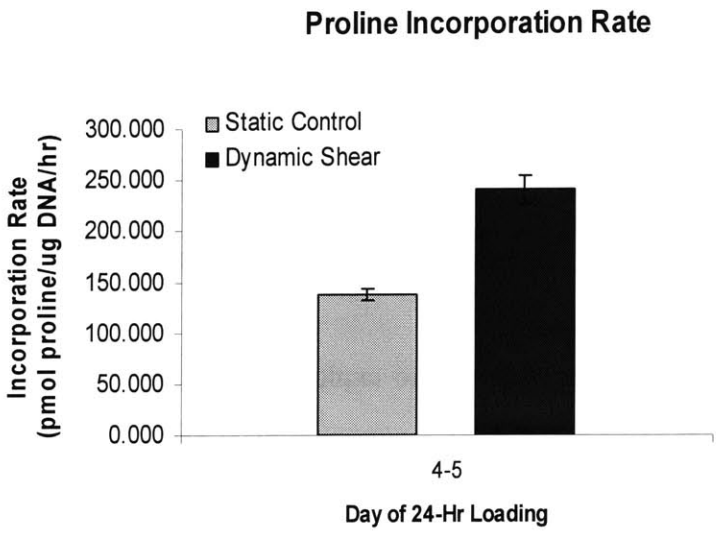



**Figure 3.2** The proline incorporation rate during the 24-hr loading period normalized to the DNA concentration of the respective loading condition. (n=12)

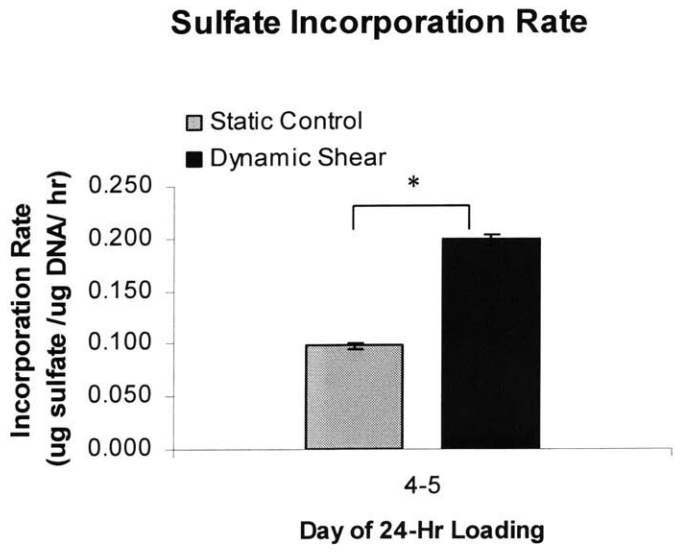



**Figure 3.3** The sulfate incorporation rate during the given 24-hr loading period normalized to the DNA concentration of the respective loading condition. (n=12)

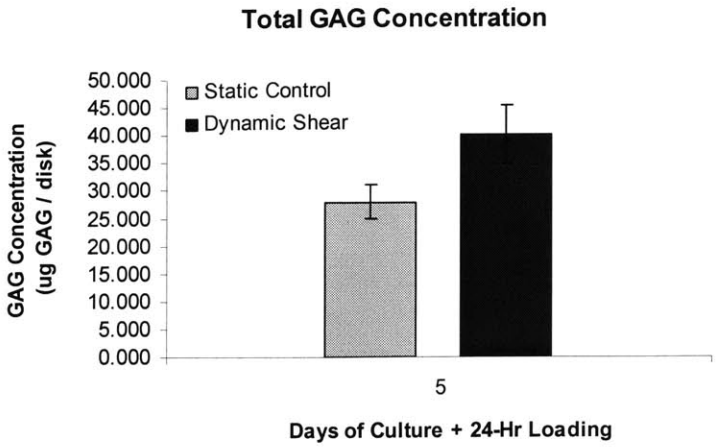



**Figure 3.4** The total accumulated GAG concentration of a disk throughout the given days of culture + 24-hr loading. (n=12)

According to Figure 3.1, dynamic shear loading merely caused a ~4% increase in DNA concentration compared to static control on day 5. Furthermore, both the sulfate (Figure 3.2) and proline (Figure 3.3) incorporation rates, indicating new protein and GAG synthesis respectively, appeared to have doubled after the cell underwent 24 hours of dynamic shear loading compared to static compression. Moreover, the total GAG synthesis after subjecting to dynamic shear loading was 31.3% more compared to static compression control, as presented in Figure 3.4.

### 3.1.2 Experiment II: can 10% FBS be replaced by 1% ITS?

In the previous experiment, dynamic shear force positively influenced matrix biosynthesis when the cell-gel slab was cultured in medium supplemented with 10% FBS. However, the goal of the 2$^{nd}$ experiment was to test whether substituting 1% ITS for 10% FBS could produce similar results and if matrix density played any role in biosynthesis. The graphs displayed below are the DNA concentration and accumulated GAG synthesis through the days of culture and applied loading, along with sulfate incorporation rate during loading.

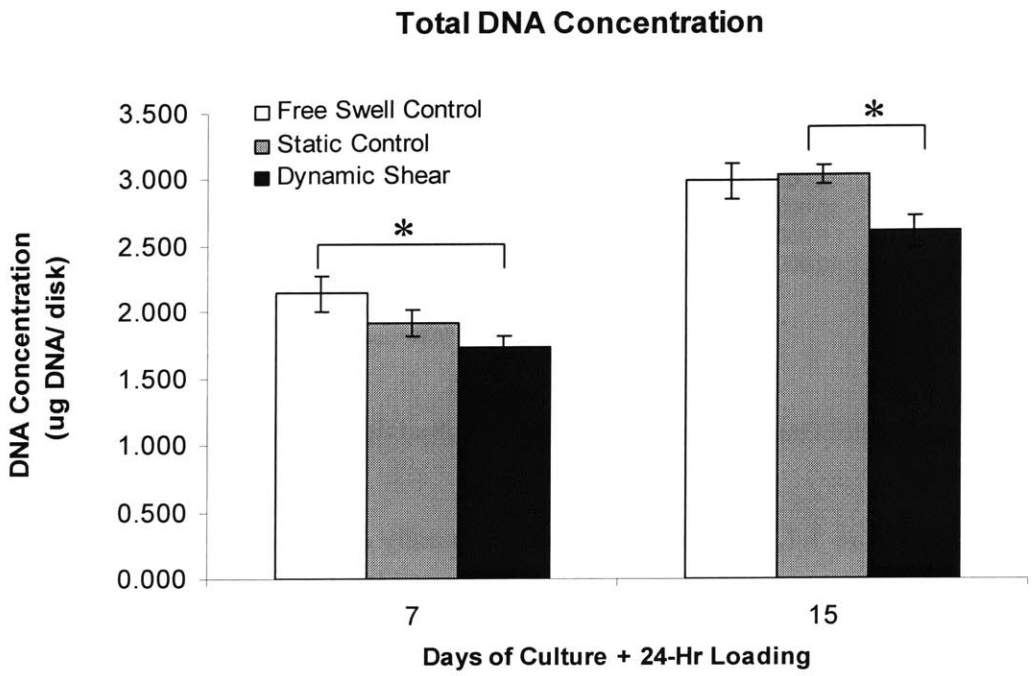



**Figure 3.5** The total DNA concentration for one disk throughout the given days of culture + 24-hr loading. (n=12)

First, cell proliferation was evident between day 7 and 15 as demonstrated by the ~50% increase in total DNA concentration between the two days, presented in Figure 3.5. Furthermore, the data showed that shear loading decreased the DNA content by ~13% compared to the controls for either time points. In fact, the decrease was statistically significant ($p<0.05$) between free swell control and dynamic shear on day 7, as well as between static control and shear on day 15. It was possible that environmental variables in the incubator housing loading apparatus contributed to the cell viability during the loading.

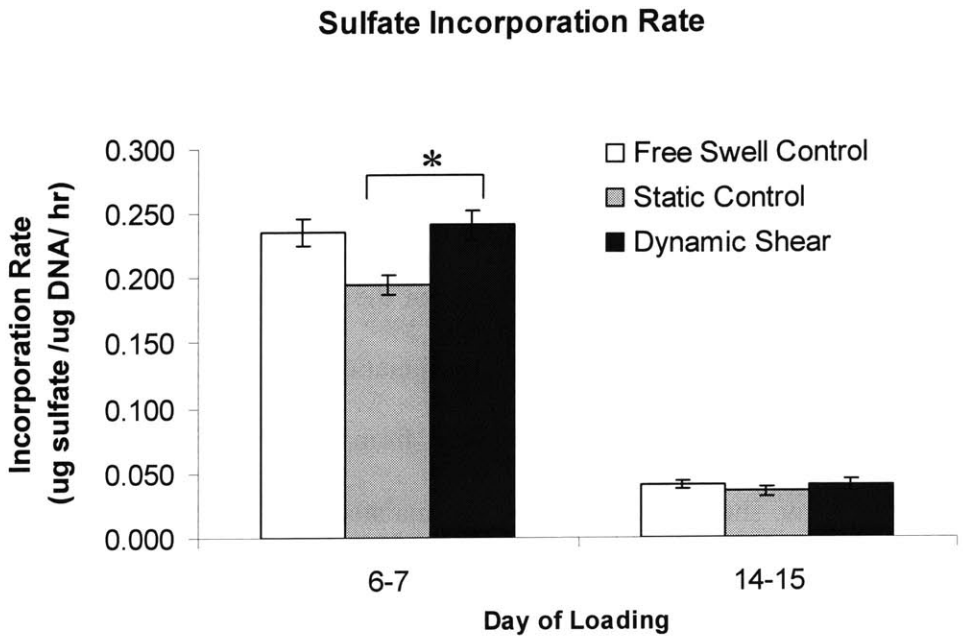



**Figure 3.6** The sulfate incorporation rate during the given 24-hr loading period normalized to the DNA concentration of the respective loading condition. (n=12)

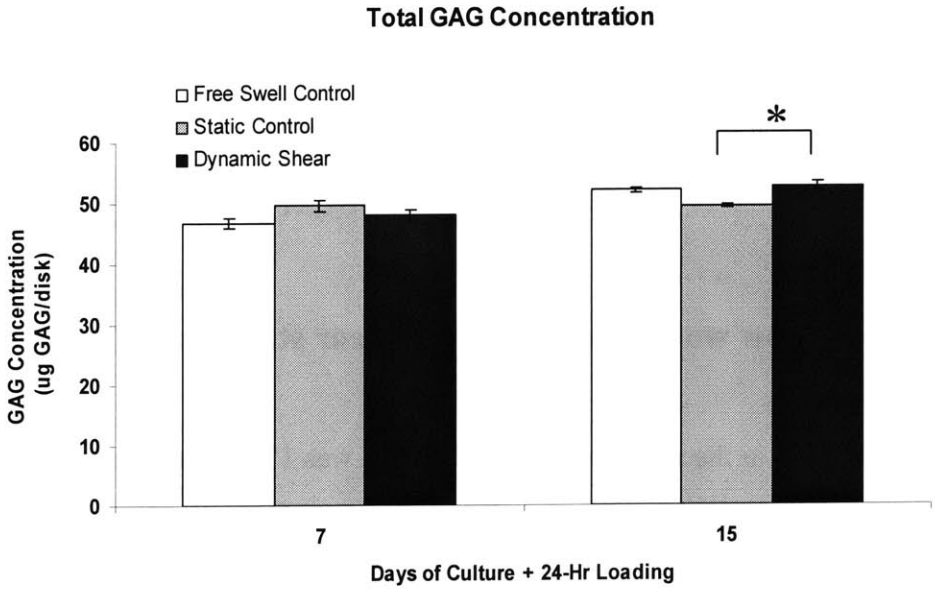



**Figure 3.7** The total GAG concentration for one disk throughout the given days of culture + 24-hr loading. (n=12)

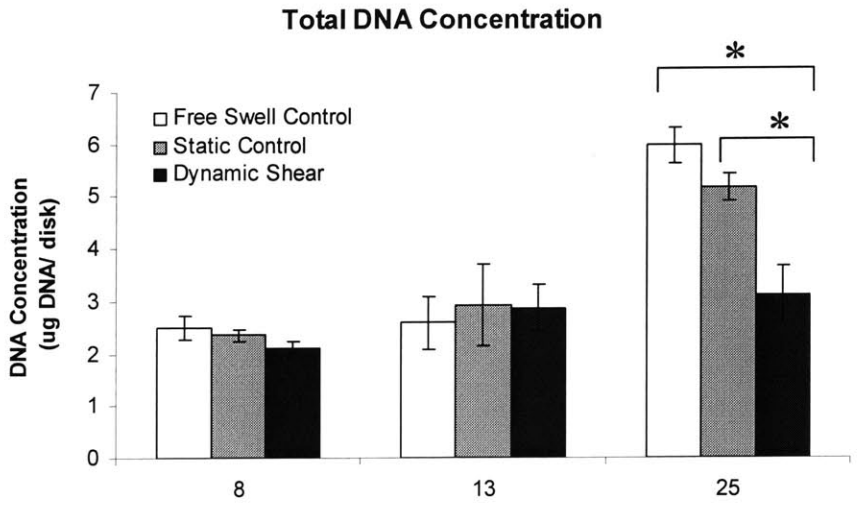
From Figure 3.6, the sulfate incorporation rate, regardless of loading condition, was approximately four times higher when the disks were loaded during day 6-7 than during day 14-15. Moreover, the GAG synthesis after disks were subjected to dynamic shear loading was ~7% lower compared to the free swell control and 19% ~ 28% higher than the static controls for both days. These findings suggest that perhaps when the matrix density was low, chondrocyte biosynthesis would occur at a much faster rate than when the matrix had permeated through much of the scaffold. According to Figure 3.7, cells proliferation did not result in a noticeable incline in total GAG accumulation retained in the disk from day 7 to15. In addition, the contribution of 24-hr dynamic shear loading to the accumulated GAG content throughout the days of culture and loading was minimal. More specifically, shear loading during day 6-7 caused a 2.67% increase from free swell control and 3.26% decrease from static control. On day 15, dynamic shear loading caused a 1.37% increase from free swell control and a 6.79% increase from static control, only the latter being statistically significant.

Meanwhile, the subtle change in GAG accumulation between day 7 and 15 suggested that there might be a sharp decrease in GAG biosynthesis rate immediately after day 7. The data for these two time points would be repeated in later experiments for verification.

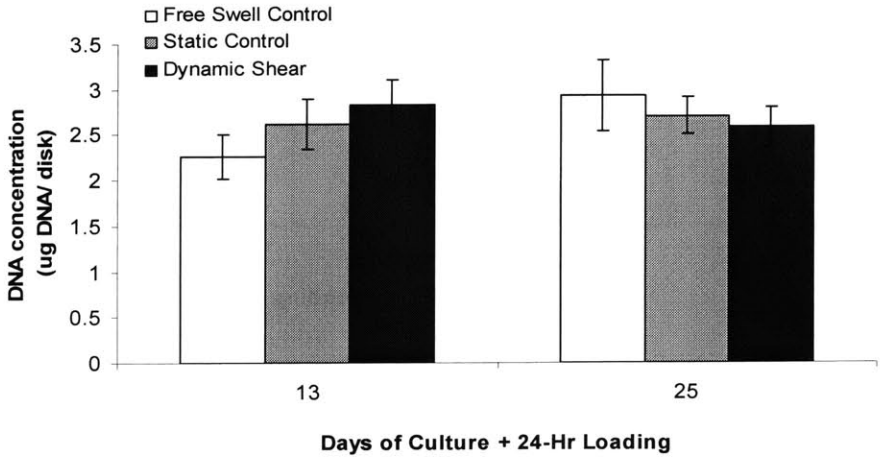
### 3.1.3 Experiment III: how would increasing the shear strain amplitude affect the result?

The angular strain used in the previous two experiments was 1%. Thus, the objective of this experiment was to check if a higher angular strain, 3% angular displacement, would further augment biosynthetic activity compared to the controls [13]. Along with that, a later loading time period, day 24-25, was selected to determine when the biosynthesis rate during the 24 hours of loading would reach a steady state. Because the biochemical assays done the first time

(shown in part A of the graphs) revealed somewhat unexpected findings, a repeat of the experiment (shown in part B of the graphs) was done in order to verify the observations.

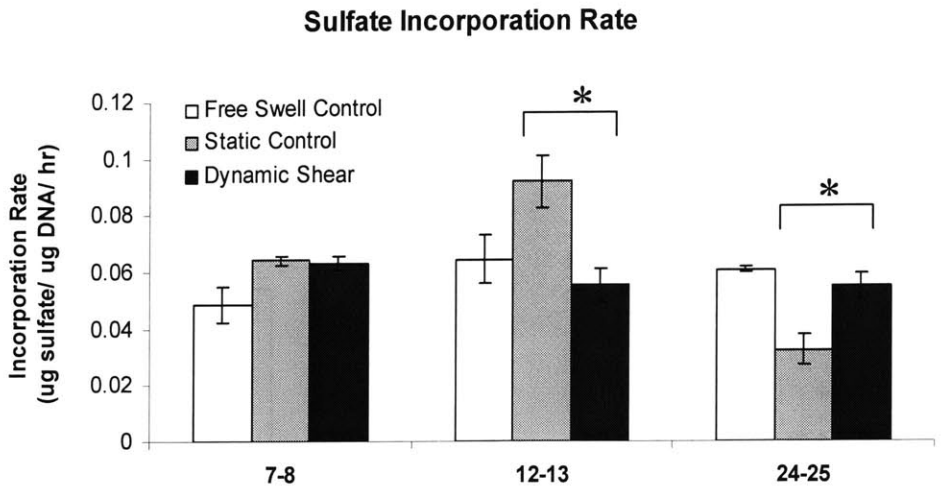



A.

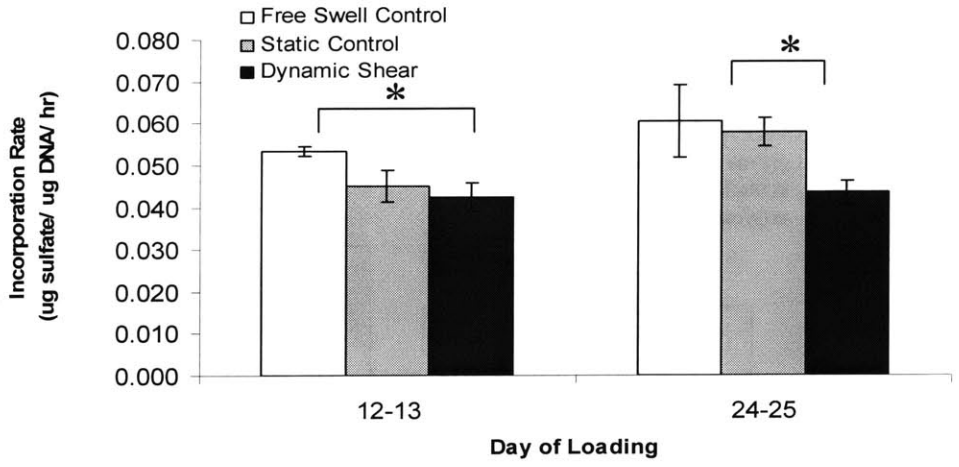



B.

**Figure 3.8** The total DNA concentration for one disk throughout the days of culture + 24-hr loading for (A) three time points and (B) two time points. (n=6)

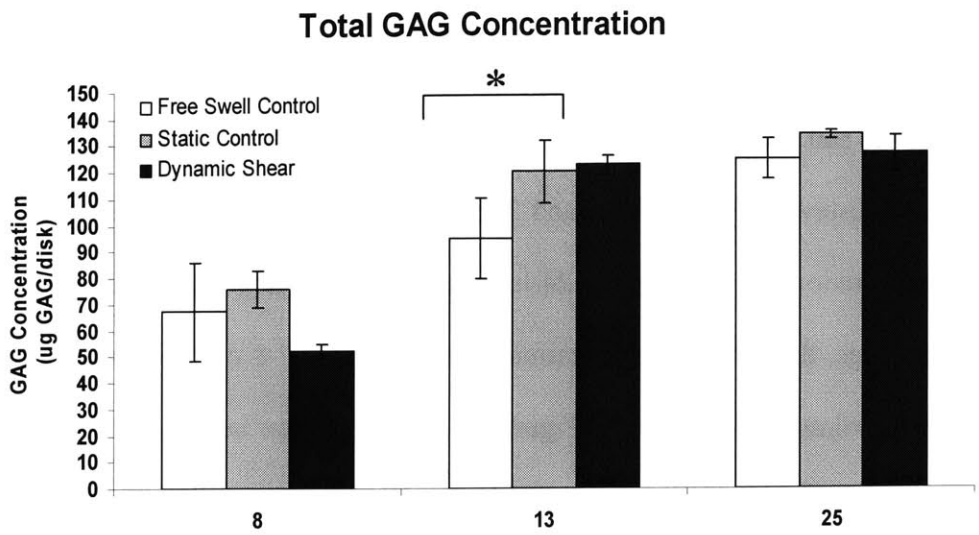



A.

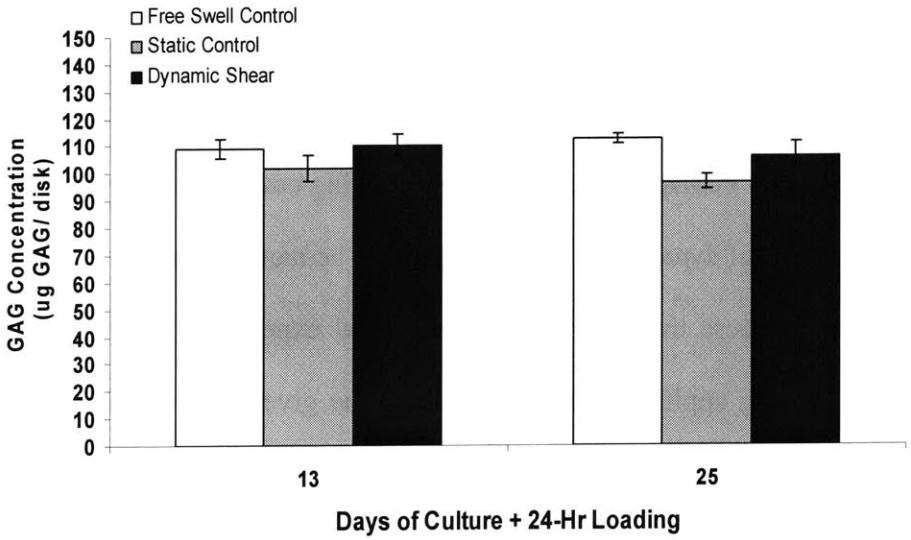



B.

**Figure 3.9** The sulfate incorporation rate during the given 24-hr loading normalized to the DNA concentration of the respective loading condition for (A) three time points and (B) two time points. (n=6)




A.




B.

**Figure 3.10** The total GAG concentration for one disk throughout the days of culture + 24-hr loading for (A) three time points and (B) two time points.

According to Figure 3.8A and 3.8B, at any given day, there was no statistical significance in the difference between DNA concentrations of disks under the various loading conditions. Also, very little cell division took place between the the days, which was unexpected.

From observing Figure 3.9A and 3.9B, dynamic shear loading negatively affected the sulfate incorporation rate during the 24-hr loading at any given loading period. Contrary to previous findings, the sulfate incorporation rate during day 7-8 did not appear to be faster than the later time points as illustrated in Figure 3.9A. The reason behind this finding could be the nearly 50% medium loss in the shear chamber wells when the disks underwent dynamic shear loading in the incubator housed loading apparatus.

Except for the day 12-13 time point on Figure 3.10A, there did not seem to be statistical difference in the total accumulated GAG concentration among the loading conditions at any given day shown on Figure 3.10A, and 3.10B. As mentioned in the previous two experiments, the subtle variation was due to that dynamic shear loading was only applied to the disks during the last 24-hr period. Perhaps if dynamic shear loading continued intermittently over a longer time frame, the effect of dynamic shear loading would be more prominent.

The data for these time points required a final experiment for verification because the dynamic shear loading applied to the disks during the given times periods were conducted in different incubator housed loading apparatus, which added a degree of variability to this experiment. In addition, the medium loss in the shear chamber could have influenced the data.

## 3.2 Final Results

### 3.2.1 Experiment IV: can 0.2% FBS make a difference in the result?

The data from Experiment I revealed that dynamic shear loading enhanced chondrocyte biosynthesis when the disks were cultured in medium supplemented with 10% FBS, as demonstrated by the doubling in sulfate and proline incorporation rates compared to the controls during the loading period. Thus, the purpose of this experiment was to see if the presence of 0.2% FBS + 1% ITS can also amplify the effect of dynamic shear loading on GAG synthesis as opposed to using only 1% ITS. At the same time, this experiment will serve as a validation for experiment III on how dynamic shear loading affected biosynthesis over two time points with angular strain amplitude of 3%. The results presented would show the respective biochemical assay data performed on day 7, 15, and consolidating the data points that only pertained to the disks that were kept in 1% ITS supplemented medium in order to compare with findings in previous experiments.

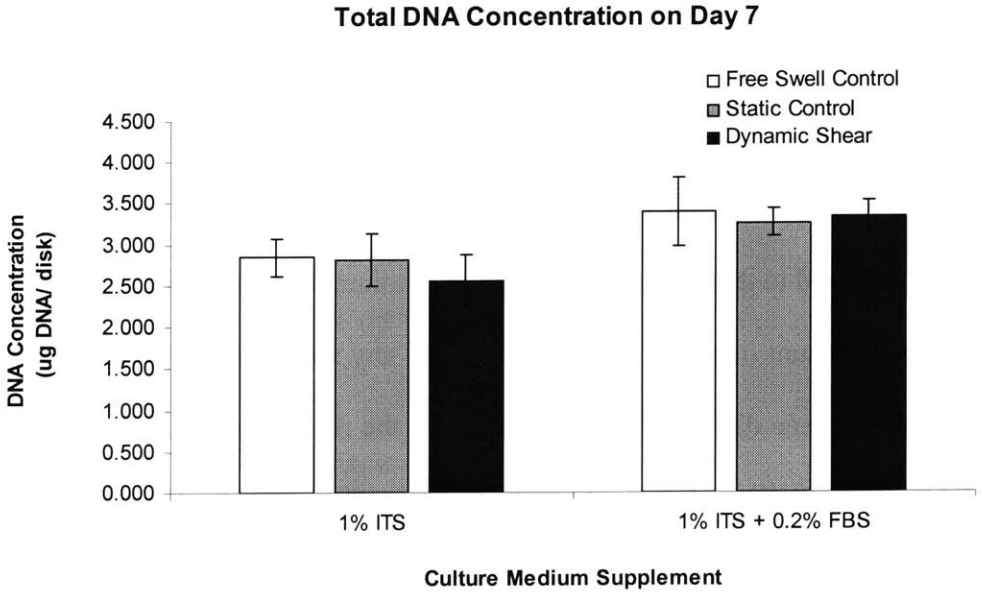



**Figure 3.11** The total DNA concentration of one disk (cultured in medium with the given supplements) on day 7. (n=6)

On day 7, when the matrix was steadily forming, the additional 0.2% FBS present in the medium caused a general increase of 16~30% in cell count compared to having only 1% ITS present according to Figure 3.11. Furthermore, the effect of dynamic shear loading on cell proliferation seemed to be similar between the two culture medium conditions.

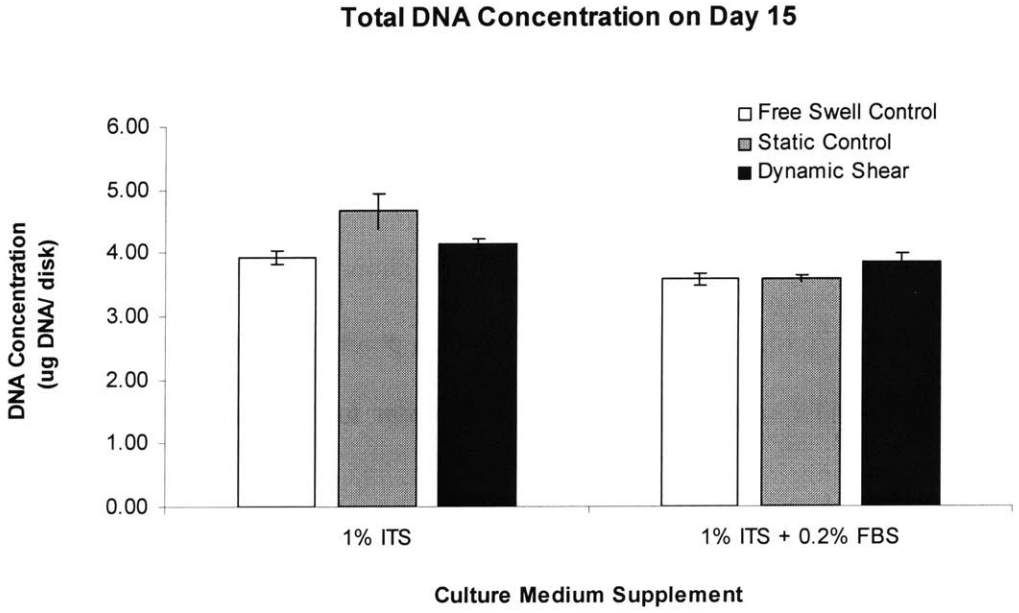



**Figure 3.12** The total DNA concentration of one disk (cultured in medium with the given supplements) on day 15. (n=6)

Then, on day 15, Figure 3.12 indicated that the presence of FBS caused a slight decrease ranging from 9~23% compared to disks that were loaded in medium that was supplemented with only 1% ITS. The small amount of serum also did not relate any statistical difference in DNA concentration on day 15 between disks that were subjected to the respective loading conditions. The increase in DNA concentration due to shear dynamic loading was ~7.7% compared to controls in the presence of 0.2% FBS. Without serum, dynamic shear loading increased the cell count by ~5% compared to free swell control.

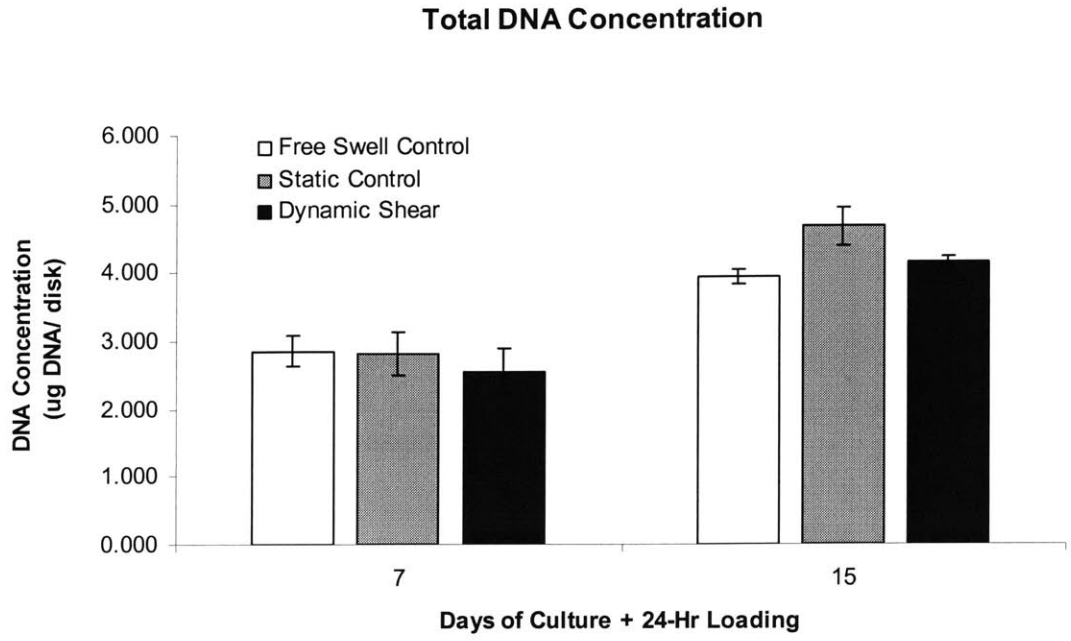



**Figure 3.13** The total DNA concentration for one disk (cultured in medium supplemented with 1% ITS) throughout the given days of culture + 24-hr loading. (n=6)

The data in Figure 3.13 pointed out that there was apparent cell proliferation that took place between day 7 and 15 with an increase of 38.3% ~ 65.6%. Similar to graph shown in Figure 3.5, there was a tiny decrease in DNA content compared to the controls after the disks underwent dynamic shear loading on day 7. Moreover, there was no statistically significant difference in DNA concentrations due to the three loading conditions.

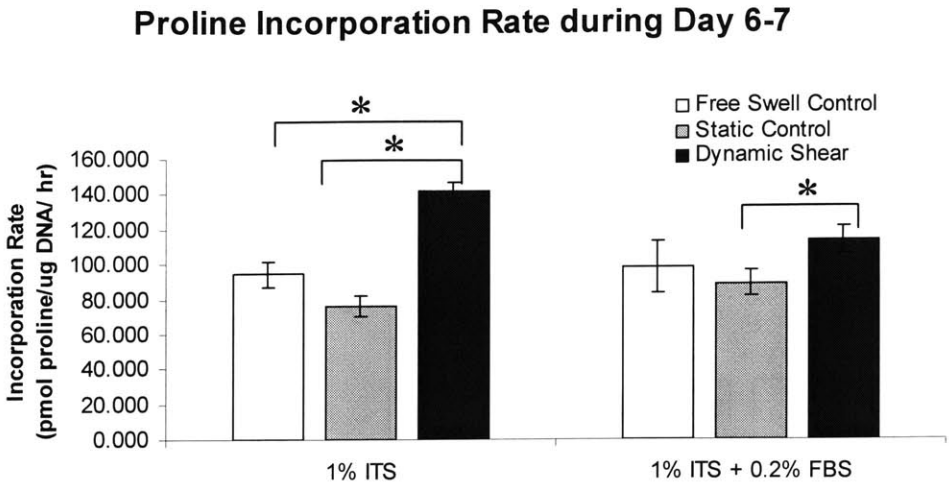



**Figure 3.14** The proline incorporation rate of one disk (cultured in medium with the given supplement) normalized to the DNA concentration of the respective loading condition for day 6-7 (n=6).

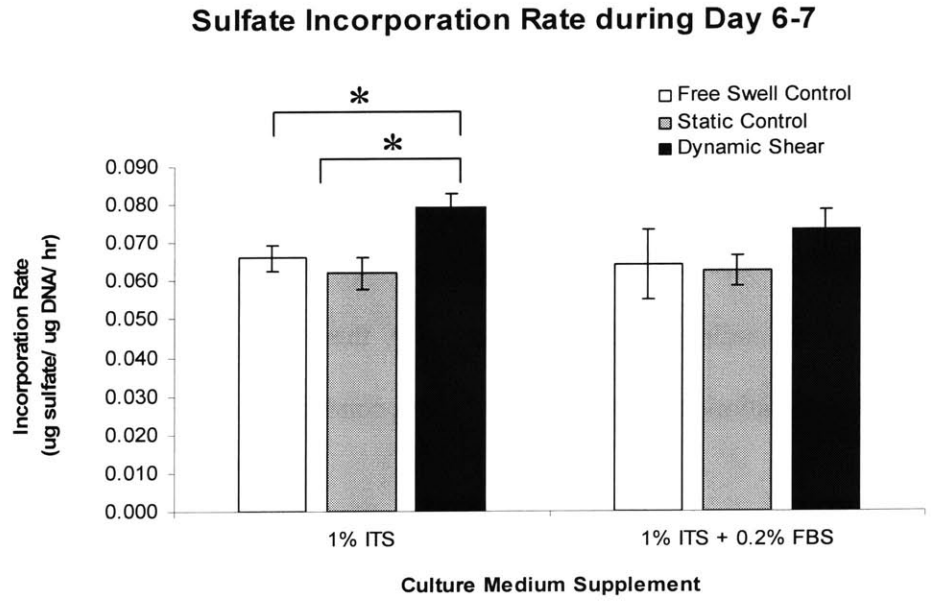



**Figure 3.15** The sulfate incorporation rate of one disk (cultured in medium with the given supplement) normalized to the DNA concentration of the respective loading condition for day 6-7 (n=6).

Both the proline (Figure 3.14) and sulfate (Figure 3.15) incorporation rate during 24-hr dynamic shear loading from day 6-7 emphasized that dynamic shear loading led to increased protein and GAG synthesis in the presence or absence of serum. In fact, the increase in the synthesis rates between the controls and dynamic shear loading was generally statistically significant when the medium was only supplemented with 1% ITS. More specifically, dynamic shear loading caused protein (Figure 3.14) and GAG (Figure 3.15) synthesis rates to increase by 51~87.4% and 20.1~27.9%, respectively, compared to the loading controls when the disks were in 1% ITS supplemented medium. However, when 0.2% FBS was present in the medium, dynamic shear loading caused protein (Figure 3.14) and GAG (Figure 3.15) synthesis rates to increase by 15.2~27.2% and 14.5~17.8%, respectively, compared with the controls. This observation addressed the fact that the presence of serum did not necessarily intensify the effect of dynamic shear loading on chondrocyte biosynthesis, at least not when the matrix formation was in its early stage around day 6-7.

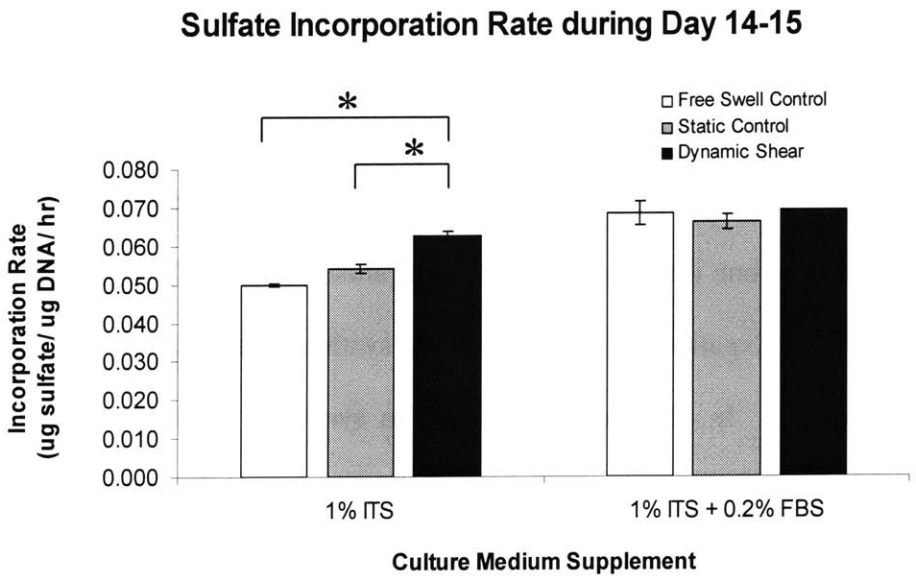



**Figure 3.16** The sulfate incorporation rate of one disk (cultured in medium with the given supplement) normalized to the DNA concentration of the respective loading condition for day 14-15 (n=6).

During the 24-hr loading period during day 14-15, the GAG synthesis rate was 15.6~25.9% higher when the disks underwent dynamic shear loading in medium supplemented only with 1% ITS, whereas the rate increase was only 0.8~4.4% in the presence of 1% ITS+0.2% FBS (Figure 3.16).

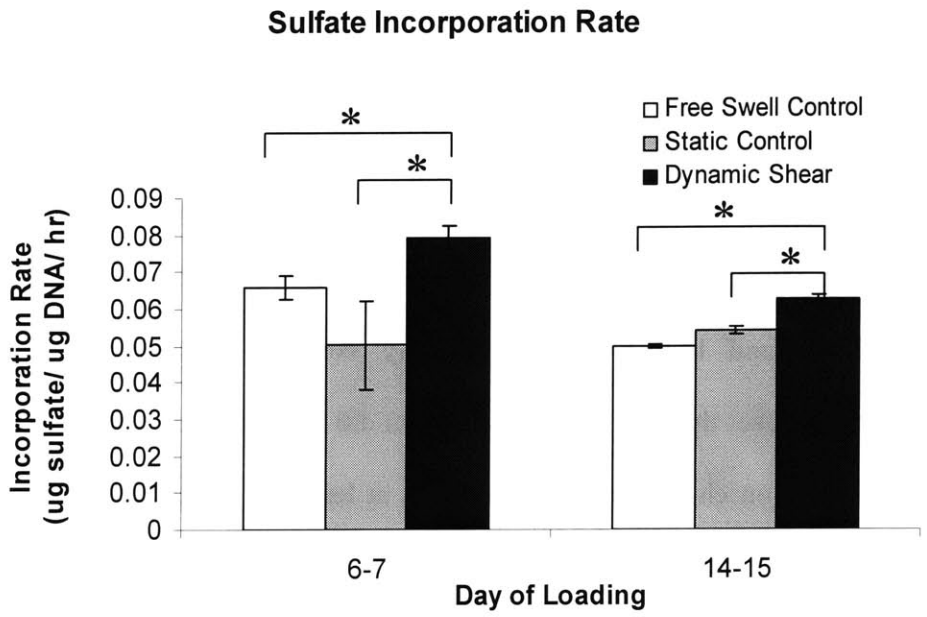



**Figure 3.17** The sulfate incorporation rate of one disk (cultured in media supplemented with 1% ITS) normalized to the DNA concentration of the respective loading condition for the given 24-hr loading period. (n=6)

Comparing the sulfate incorporation rate between the two loading time periods, denoted by day 6-7 and 14-15, the overall decline in GAG synthesis rate was around 21% as matrix formation progressed from one week to two weeks in agarose culture. This finding, along with the similar observation in Experiment II, suggested that synthesis was higher when loading was applied at an earlier time. In addition, the increase in synthesis rate due to dynamic shear loading reaffirmed the prediction that shear force could constructively impact the GAG and protein synthesis during the first two weeks of matrix generation.

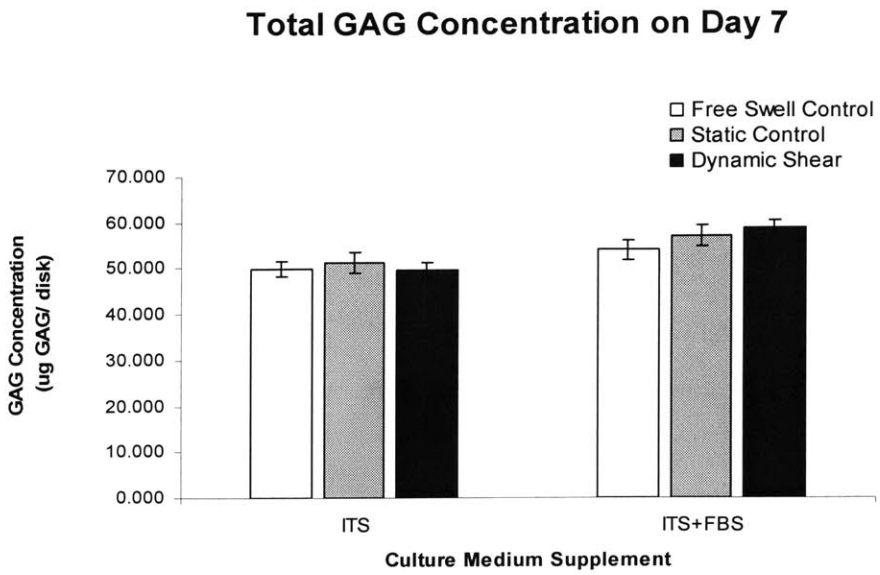



**Figure 3.18** The total GAG concentration of one disk (cultured in medium with the given supplements) on day 7. (n=6)

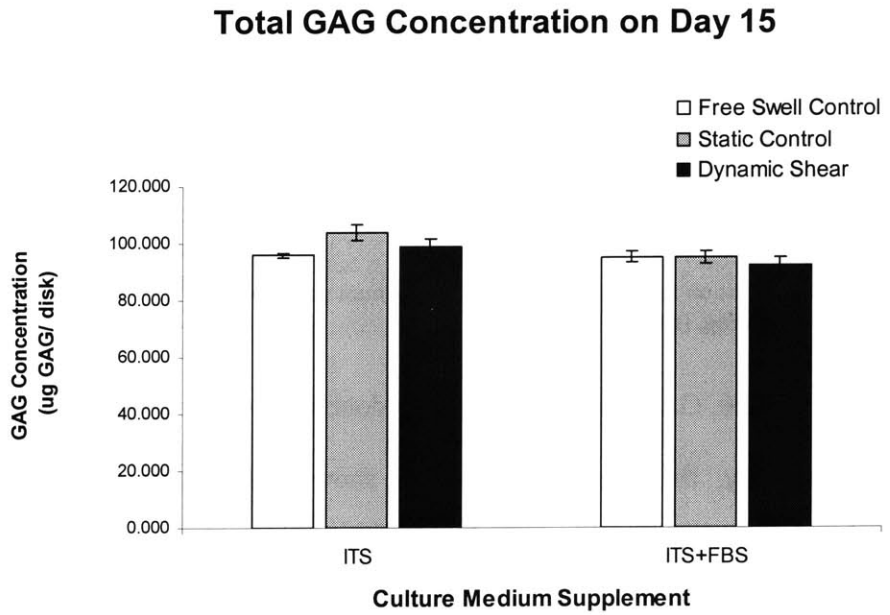



**Figure 3.19** The total GAG concentration of one disk (cultured in medium with the given supplements) on day 15. (n=6)

The presence or absence of serum in medium caused the total accumulated GAG concentration, shown in Figure 3.18 and 3.19, to differ by 12% and 5.5%, respectively, on day 7 and day 15. Furthermore, the effect of the merely 24-hr dynamic shear loading was overshadowed by the scale of the total GAG synthesis in the case of either medium supplement.

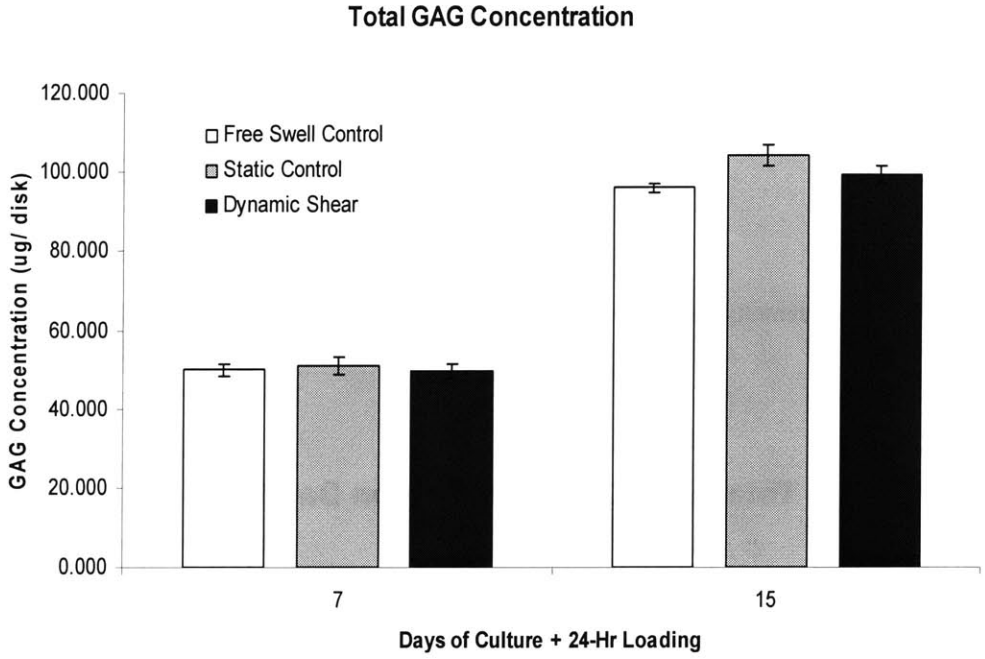



**Figure 3.20** The total GAG concentration for one disk (cultured in medium supplemented with 1% ITS) throughout the given days of culture + 24-hr loading. (n=6)

According to Figure 3.20, GAG content had nearly doubled between day 7 and day 15 as expected. On any given day, the biochemical assays showed that neither culture media supplement was more effective in influencing the effect of shear loading on chondrocyte biosynthesis.

### 3.2.2 Experiment V: is there an earlier time point that would yield the optimal result?

Since previous data had demonstrated that biosynthesis rate was higher when shear loading was applied on day 6 instead of day 14, this experiment intended to explore the possibility that there was an even earlier time point that would provide the optimal results. Therefore, the time course selected for this experiment began with day 0, which was the day that the chondrocytes were encapsulated inside the agarose gel. Subsequent time points included day 2, 4, and 6 with small intervals apart, and the last one was reserved for day 14. Once again, biochemical assays were assessed to understand at what stage of the matrix could shear loading have more impact on the synthesis.

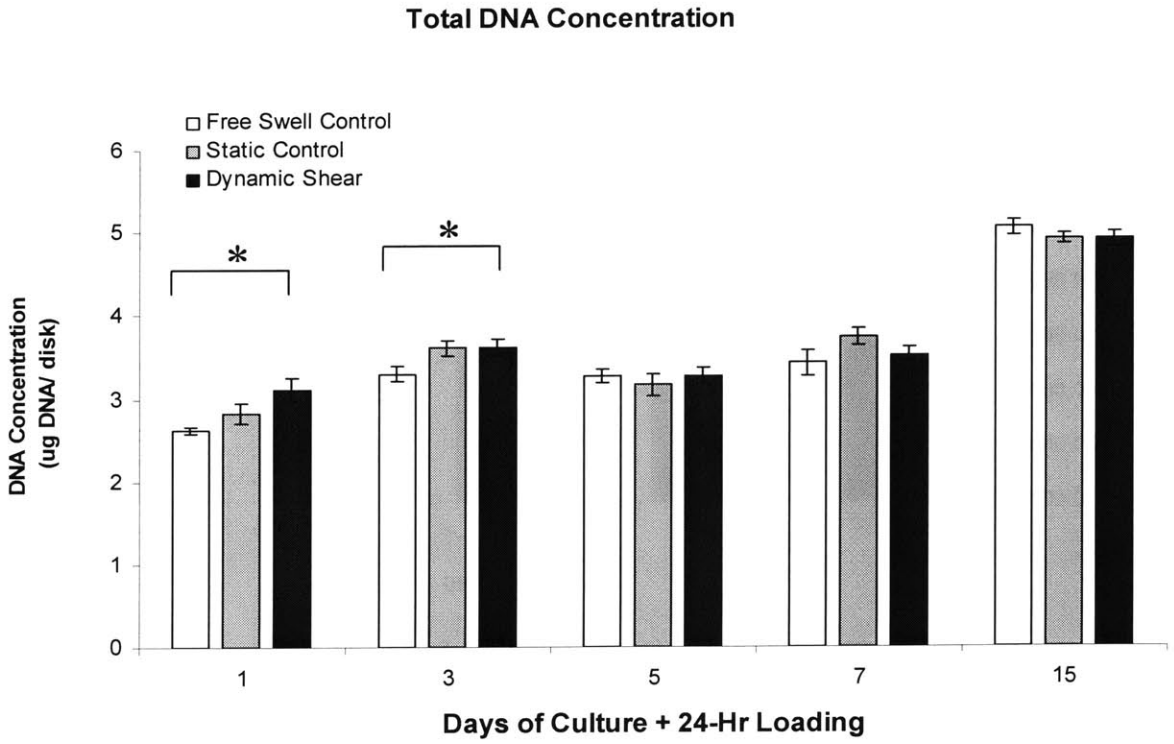



**Figure 3.21** The total DNA concentration for one disk (cultured in medium supplemented with 1% ITS) throughout the given days of culture + 24-hr loading. (n=6)

As shown in Figure 3.21, the DNA concentration remained relatively constant from day 1 through day 7. However, there was a drastic increase in DNA concentration from day 7 to day 15, indicating a great amount of cell proliferation, similar to the case in experiment IV as referred by Figure 3.13. Consistent with previous findings, the effect of 24-hr shear loading on the accumulated cell number was not very pronounced in this graph except for on day 1 and 3 where the DNA concentrations were shown to be significantly different between dynamic shear and controls.

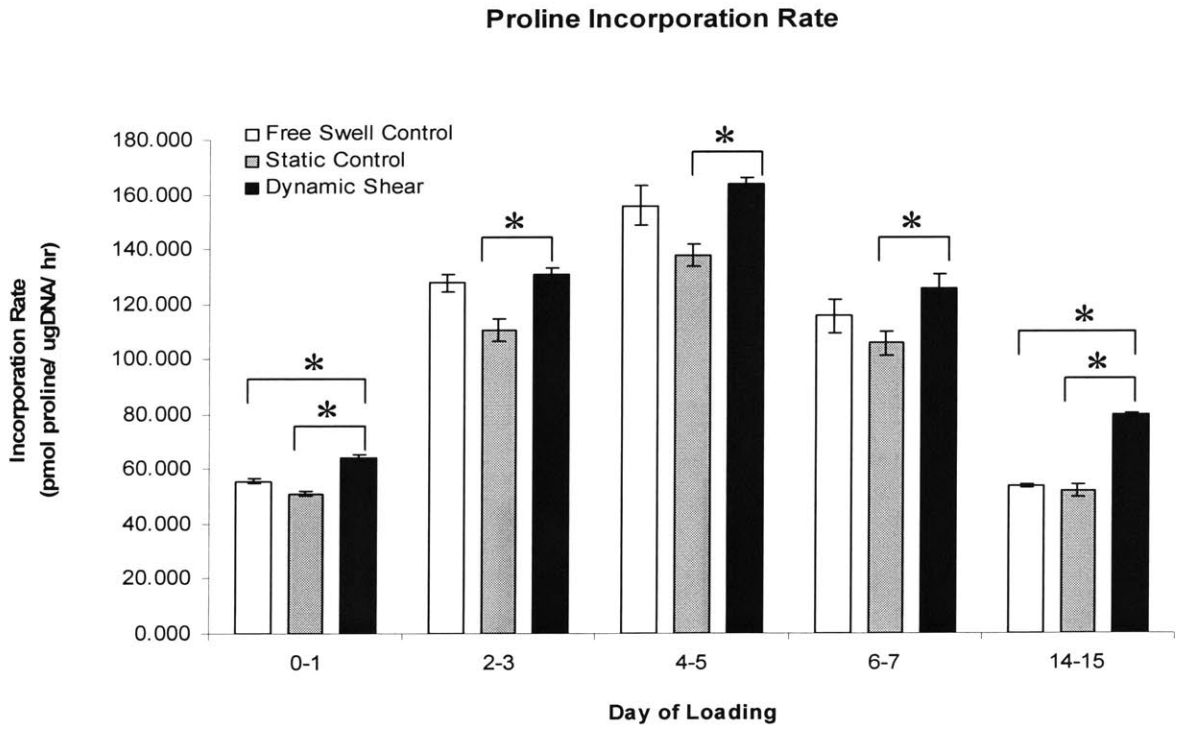



**Figure 3.22** The proline incorporation rate of one disk (cultured in media supplemented with 1% ITS) normalized to the DNA concentration of the respective loading condition for the given 24-hr loading period. (n=6)

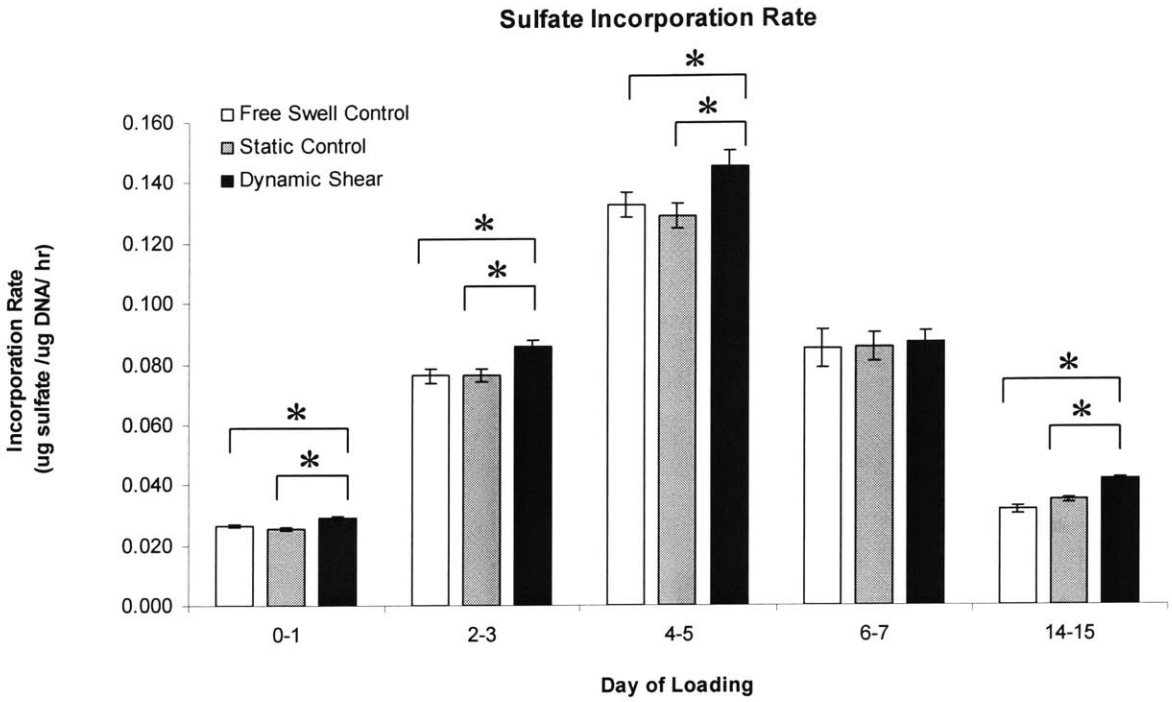



**Figure 3.23** The sulfate incorporation rate of one disk (cultured in media supplemented with 1% ITS) normalized to the DNA concentration of the respective loading condition for the given 24-hr loading period. (n=6)

The trend for proline (Figure 3.22) and sulfate (Figure 3.23) incorporation rates during the first two weeks were quite similar. In both cases, the initial incorporation rates started slow. Then, as time passed, chondrocytes biosynthesis steadily sped up until an apex was reached during the day 4-5 of loading. Then, both rates decayed to a steady state probably around the second week. Moreover, on any given day, 24-hr continuous dynamic shear loading led to a small, yet statistically significant increase in protein and GAG synthesis. Although that difference was not statistically significant on day 6 for this particular joint, it was safe to infer from previous experimental findings that dynamic shear loading could cause a prominent increase in biosynthesis rate during day 6-7.

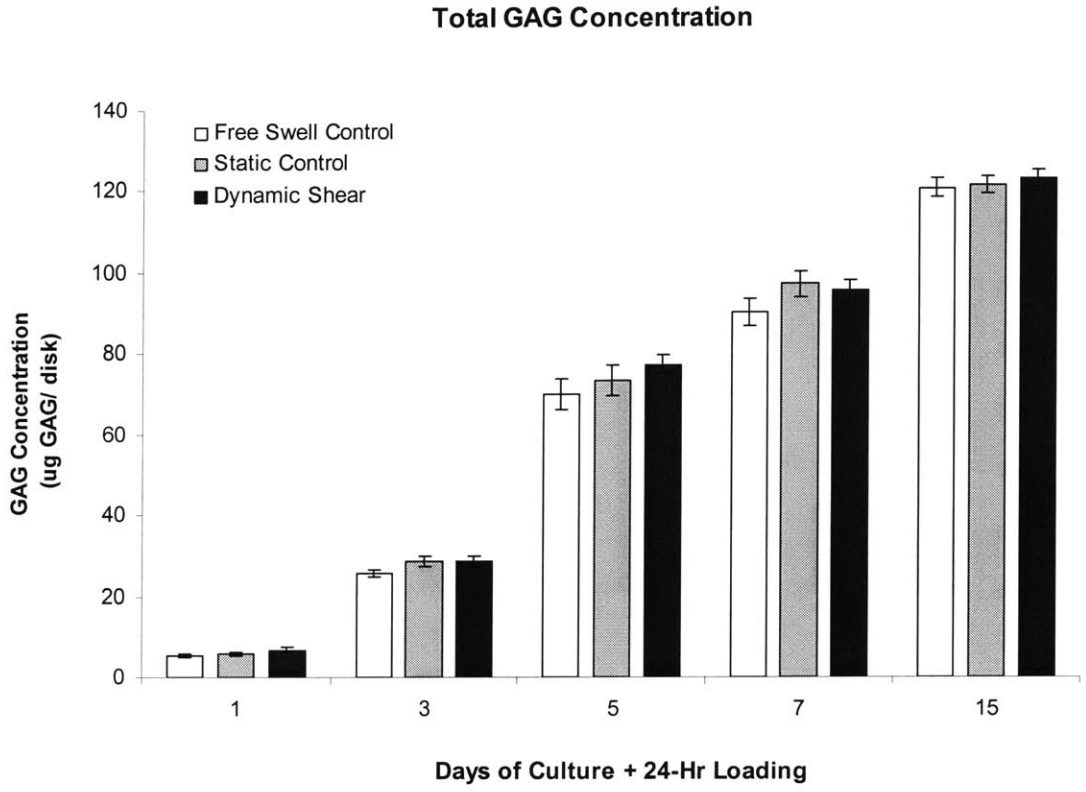



**Figure 3.24** The total GAG concentration for one disk (cultured in medium supplemented with 1% ITS) throughout the given days of culture + 24-hr loading. (n=6)

As expected, Figure 2.24 showed that the accumulated GAG concentration retained in the disk increased steadily. However, although 24-hr dynamic shear loading effect during the last day was miniscule seen from the graph, longer duration of intermittent loading could further enhance the difference in synthesis.

# Chapter 4

# Discussion

## 4.1 Overall Analysis

The fundamental conclusion that can be drawn from all the experiments presented here is that dynamic shear loading can stimulate chondrocyte biosynthesis, as quantified by the GAG and protein content. Based on the time course data, at any given time, dynamic shear loading caused statistically significant increases in chondrocyte synthesis of GAG and total protein. Since ~75-80% of the $^{3}$H-proline is incorporated into collagen in those newborn calf cartilage explants, we can conclude that shear loading increased collagen synthesis [27]. It is difficult to gauge whether dynamic shear deformation favors synthesis of collagen over GAG in agarose culture because it seems that both rates increase compared to controls by a similar percentage after 24-hr dynamic shear loading. In the process, we also learned that there are many factors which play a role in optimizing the effect of dynamic shear loading on chondrocyte biosynthesis. These factors include the existing state of the cell-gel construct, the supplements added to the culture medium, and the shear loading protocol.

### 4.1.1 State of Cell-Gel Construct

From the results presented in the Experiment II-V, sulfate and proline incorporation rates during loading depended on the day on which loading was performed. In Experiment II and III, it was clear that the synthesis rate was higher when 24-hr shear loading was applied sometime following the first week after casting rather than the second or third week. This observation

implies that perhaps the mechanical signal sent out by shear loading can be better received by chondrocytes when they are just beginning to be surrounded by PCM and some further removed ECM in the early stage of development. Also, the fact that biosynthesis was low when cells were first introduced into agarose culture during the first 1-3 days and steadily increases as more PCM is deposited around them further reinforces a previous finding that the presence of PCM enables higher matrix synthesis rate than chondrocyte alone [28]. Eventually, as the ECM further develops within the agarose culture, chondrocyte response to shear appears diminished.

Additionally, according to the data from experiment V, the biosynthesis rate for all the loading cases peaked during day 4-5 and minimal cell proliferation occurred between day 1~7. If that pattern holds true, then somewhere between day 7 and 14 when cells are more actively dividing, there might be another crest in biosynthesis rate that pertain to the new cells. So, it would be worthwhile to observe additional time points in between day 7 and 14.

### 4.1.2 Medium Supplement

Throughout the experiments, three supplements were added into the cultural media: 10% FBS, 1% ITS, or 1% ITS + 0.2% FBS. As noted in experiment I, the effect of loading on biosynthesis rate was definitely apparent when the medium was supplemented with 10% FBS. Yet, 1% ITS and 1% ITS + 0.2% FBS were shown in experiment IV to have similar effects on matrix biosynthesis for all loading cases. Perhaps, this result can be attributed to the lack of a combined loading and serum growth factor effect. The analogous reasoning for that being IGF-I and dynamic shear force have been shown to independently affect biosynthesis in cartilage explants [29]. Another possibility is that since the additional 0.2% FBS did not affect biosynthesis within chondrocyte-agarose construct under free swell condition, maybe it would

not enhance the effect of dynamic shear force on matrix biosynthesis either [24]. Thus, the findings of experiment IV suggest that low concentration serum of serum does not aid dynamic shear loading in speeding up matrix synthesis.

### 4.1.3 Loading Protocol

Based on all the total GAG data, the effect of 24-hr dynamic shear loading was overshadowed by the existing accumulated GAG within the construct. However, the increase in biosynthesis rate due to dynamic shear loading during the 24-hr period was quite distinct. Therefore, theoretically, it is possible that longer, intermittent shear loading protocols can be applied to the disks to enhance the cumulative biosynthesis.

Additionally, according to results from experiment III, increasing the shear displacement amplitude from 1% to 3% did not evoke much change in biosynthesis.

## 4.2 Future Directions

For more extensive studies surround shear loading effect on biosynthesis, it would be interesting to repeat experiment V while looking at the time points between day 7 and 14. The motivation for this, as mentioned above, is to discover whether there is another peak value in biosynthesis rate upon new cell formation. Following that, long term intermittent shear loading effects can be investigated to see whether it can invoke a more noticeable effect in accumulated GAG than 24-hr continuous loading. For example, we can compare the following two loading protocols after culturing for 6 days: loading continuously for 24 hours versus loading intermittently for 3 days. If intermittent loading for longer period fares better than 24 hours, then the next question would be to ask: what is an optimal day to begin loading? Perhaps, loading

after four days of culture when the biosynthesis is the fastest would be a better choice than loading after 3 weeks of culture.

Finally, another parallel study would be to identify electrostatic contributions to the shear modulus of the cellular agarose construct due to GAG content.

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